

# ANNUAL REVIEW OF BIOCHEMISTRY

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VOLUME 27

1958

ANNUAL REVIEWS, INC.  
PALO ALTO, CALIFORNIA, U.S.A.



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PALO ALTO, CALIFORNIA, U.S.A.

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*Library of Congress Catalog Card Number: 32-25093*

FOREIGN AGENCY

Maruzen Company, Limited  
6, Tori-Nichome Nihonbashi  
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY  
THE GEORGE BANTA COMPANY, INC.

## PREFACE

It is appropriate that we pay tribute to the memory of Claude Fromageot, whose death on January 10, 1958, deprived France of one of its great scientists and biochemistry of one of its most distinguished investigators. Those of us who knew him personally recall his integrity of person, his warm friendliness, and the enthusiasm which was always his. He contributed much to our knowledge of enzymes and of the chemistry of proteins and peptides. At the time of his death, he was engaged in the preparation of a review on the metabolism of the sulphur-containing amino acids and related compounds. It had been his hope and ours to publish it in this volume of the *Annual Review*.

We wish to thank most warmly all who have contributed in authorship to this volume. We regret that a review on Mechanism of Enzyme Action had to be cancelled at the last moment—too late to make other arrangements for its preparation.

We wish that we could transmit adequately to the authors the thanks of those who use these Reviews, for, on the part of the contributor, the task is entirely a labor of love—of devotion to the subject. His efforts to encompass the literature are necessarily attended by increasing frustrations as the various segments of the subject continue to expand without limit. Some of the problems that are arising from the spate of the world's scientific and cultural literature have been the subject of study by many national and international conferences during the past decade, most recently by the A.A.A.S. Parliament of Science.

The Editorial Committee of the *Annual Review of Biochemistry*, which will meet in Vienna on September 2 in conjunction with the International Congress of Biochemistry, will be concerned with the vexatiously rapid growth of the periodical literature in biochemistry, a problem which is very pertinent to this *Annual Review*. The Committee actively seeks advice as to authors and topics. This advice is continuously re-appraised as potential contributors become available and new topics come to the fore. Suggestions from readers of these volumes will always be most welcome.

In thanking those who have given so generously of their time, we extend also our thanks to the editorial assistants, whose help has been indispensable. We derive much help from the staff of the business office, whose cooperation in certain phases is greatly appreciated. We express our gratitude particularly to Adele Fumino, whose responsibilities in the editorial office were focused for much of her time on this volume. Once more we thank our printers, the George Banta Company, for their unstinted cooperation.

F.W.A.	J.M.L.
F.S.D.	G.M.
W.Z.H.	E.L.S.
B.L.H.	E.S.

## ERRATA

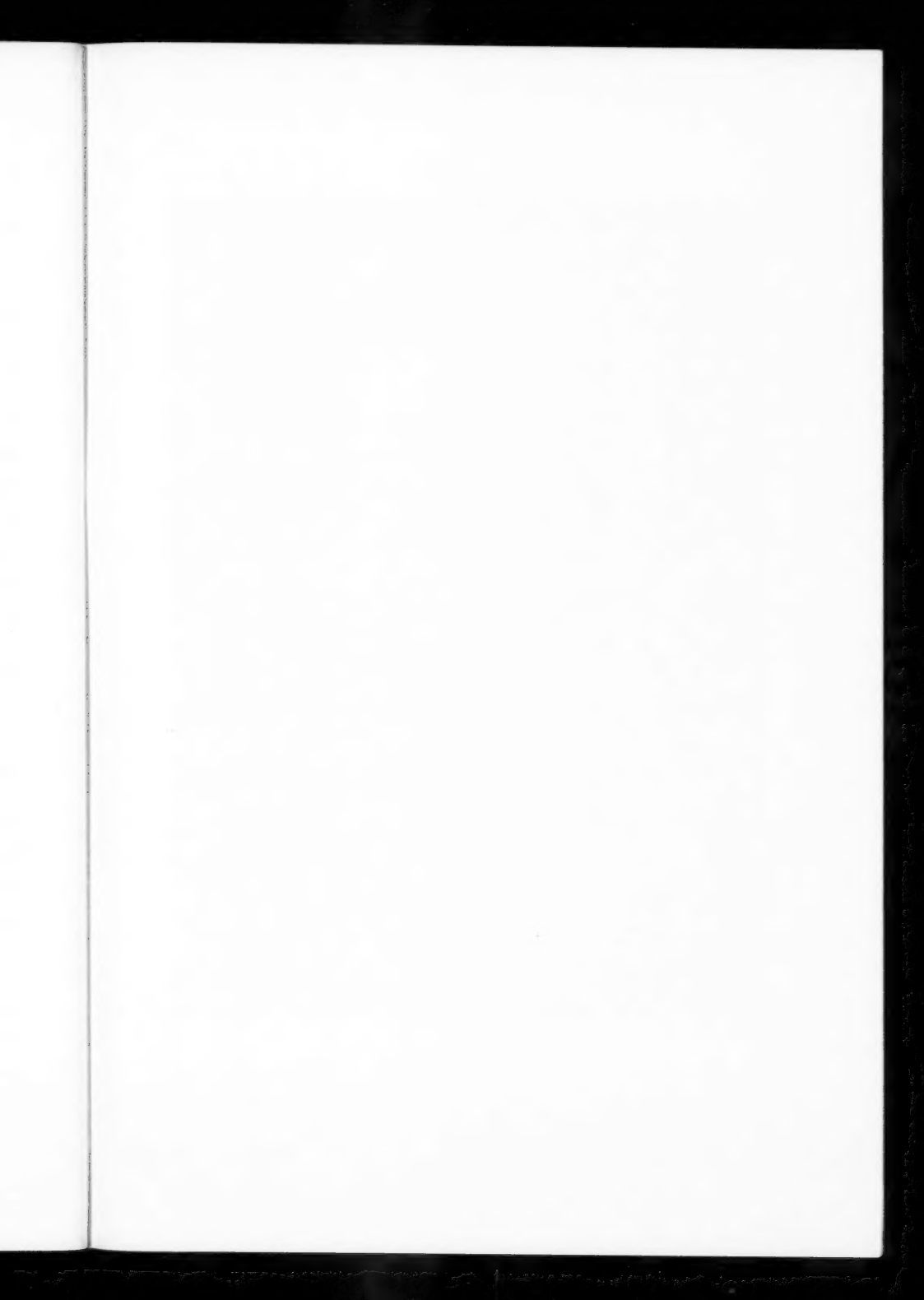
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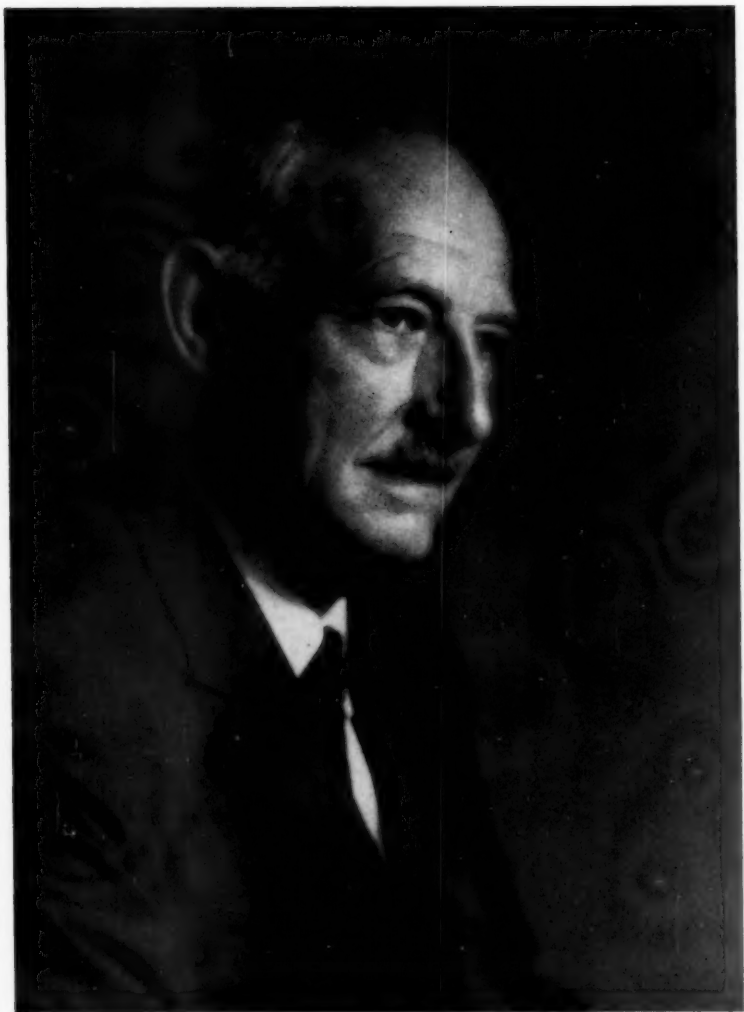
- page 77, lines 10 and 14: *for* phosphonates *read* phosphorohalidates
- page 77, line 25: *for* isomethylphosphonic acid *read* isopropyl methylphosphonic acid
- page 100, line 28: *for* pepsin *read* trypsin
- page 262, line 15: *for* and *S. Faecalis*. The inhibition indices with *read* but not of *S. Faecalis*. The inhibition indices for *L. casei* with
- page 455, lines 21 and 27: *for* ergothionine *read* ergothioneine
- page 572, lines 42 and 43: *for* 2,6 isomer *read* 5,6 isomer
- page 713, column 2, between lines 50 and 51, *insert* Henderson, R. W., 39

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Hans C. Clarke

## PREFATORY CHAPTER

### IMPRESSIONS OF AN ORGANIC CHEMIST IN BIOCHEMISTRY

BY HANS T. CLARKE

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When during the last century the early gropings were made towards the clarification of biochemical problems, these were mainly carried out by investigators whose prime interest lay in the rapidly expanding areas of organic chemistry. Their orientation was in many instances influenced by training in medicine, in which the need for wider knowledge of the processes underlying physiological and pathological phenomena had become obvious to them. As in every other branch of science, the initial phases necessarily consisted in observation and description. Early impetus to investigations of this character was given by Mulder, Chevreul, Liebig, Wöhler and Pflüger, but in the rudimentary state of organic chemical knowledge then existing, such stimulation was necessarily more general than specific in direction.

For many years progress towards biochemistry was slow, in spite of the autocatalytic development of organic chemistry, for the ramification of that discipline developed more along the lines of synthetic reactions than of the study of compounds of biological origin. A decisive change, however, was initiated towards the end of the last century by the masterly researches of Emil Fischer on the chemical structure of purines and sugars. These set the pattern for the next approach to fundamental biochemistry. Fischer's ensuing attack on the chemistry of proteins, skillful though it was, proved less fruitful than arduous. The procedure which he developed for the determination of the component amino acids of proteins yielded only approximately quantitative values and required many months of intensive labor for the analysis of a single protein; his synthetic approach, based on recognition of the peptide linkage of the components, yielded polypeptides which bore little physical resemblance to proteins.

This was the situation around 1907, when, as a prospective organic chemist studying in London under Sir William Ramsay and J. Norman Collie and taking physiology as a minor subject under E. H. Starling, I was first exposed to physiological chemistry. The instruction in this subject, which had not yet attained the status of a discipline, was given by R. H. Aders Plimmer. It included the isolation of a few crystalline proteins, carbohydrates, and amino acids; the preparation of lecithin and kephalin; and the estimation of some urinary components by procedures which had recently been developed by Folin. These exercises did not greatly appeal to me; I found them intellec-



tually far less rewarding than the rich fare offered by Collie and Smiles in their courses on organic chemistry.

In the years 1911 to 1913, I had the privilege of working in Emil Fischer's laboratory in Berlin as a research guest supported by an "1851 Exhibition" scholarship from London. According to rumor, Fischer had once taken into his group a Briton who had not worked assiduously, and he had consequently decided never again to accept a "lazy Englishman" as a collaborator. Although I worked on problems of my own, Fischer visited me almost daily in the laboratory and discussed my experiments, and many other subjects, with the greatest friendliness. It was also my good fortune there to associate with several men who later became intimate friends, particularly Roger Adams, Max Bergmann, Harold W. Dudley, and Karl Freudenberg, as well as my cousin B. Helferich, who was then Fischer's private assistant.

On my arrival in Berlin, Helferich advised me, much to my surprise, not to ask the other members of the laboratory what they were doing. This was so contrary to British tradition that I was interested to find out the reason; it appeared that most of the chemists who were working on topics of their own were retained as consultants by one or another of the German chemical manufacturing firms, which had priority on any patentable discoveries made by the individuals concerned. This system appeared to me, as it still does, as being at variance with the prime function of an academic laboratory.

After three profitable and enjoyable semesters in Berlin I spent a summer at Queen's University, Belfast, in the laboratory of my friend A. W. Stewart, and then returned to University College, London. In the early summer of 1914 I went to Rochester, New York, at the behest of George Eastman of Eastman Kodak Company, for which concern my father was in charge of technical developments in Europe. As the company at that time had no organic chemist on its staff, Mr. Eastman had occasionally sought my advice on organic chemical matters. While I was in Rochester, C.E.K. Mees, the director of the recently established research laboratory of Kodak, offered me a full-time appointment in a new section for organic chemical research and this I accepted. Owing to the outbreak of the first World War, the importation of German chemicals had been cut off, and my first tasks were to work out processes for the manufacture of photographic developing agents, and then of the laboratory preparation of sensitizers. In this last-named effort I was, after a few years, joined by L. G. S. Brooker, who has since carried forward the work independently with masterly skill.

In the summer of 1918, Mees charged me with the organization of a laboratory for the preparation of research organic chemicals to meet the urgent needs of universities, whose stocks had become depleted owing to the impossibility of securing supplies from Germany. As at that time almost all of the relatively few American-trained organic chemists were actively employed in government service, the laboratory was staffed by young women, all

recent college graduates who had majored in chemistry. These girls displayed immense enthusiasm, cooperativeness, and application, but in general were not well adapted to preparative work on a large laboratory scale; accidents were alarmingly frequent and it proved impossible to assign more than two preparations to each girl for simultaneous operation. After the first year, therefore, replacements and additions to the group were made with men. Among these was a youngster named Warren Sperry, who some ten years later again became associated with me at the Columbia-Presbyterian Medical Centre. During the past thirty years the work of the laboratory of Synthetic Organic Chemistry in Eastman Kodak Company has been directed by W. W. Hartman under whose supervision an impressive list of available organic compounds has been built up.

Around 1926 I undertook an additional assignment for the initiation of a laboratory for the exploitation of cellulose esters for photographic film. The experimental work in this field was carried out from the outset, and has subsequently been directed with great acumen, by C. J. Malm.

In these various laboratories the staffs were from time to time reinforced by young academic organic chemists who joined on a temporary basis. Of these I recall with special pleasure and admiration W. E. Bachmann and C. R. Noller, both of whom subsequently made notable contributions to organic chemistry.

In 1928, on the suggestion of H. D. Dakin, I was invited by Columbia University to direct the Department of Biological Chemistry in the College of Physicians and Surgeons. The Faculty of Medicine, in view of the trend in biochemistry, recognized the desirability of departure from the classical approach of physiological chemistry to the problems of medicine. I gladly accepted the challenge and have been happy ever since, in spite of a keen and constant recognition of my inadequacies in the field.

When I entered Columbia, the medical school had just moved to its present location on West 168 Street. The departmental laboratory, though new, was not well equipped with modern facilities; fortunately, a liberal grant from the Chemical Foundation made possible the supplementation of laboratory installations and the purchase of a truly adequate series of chemical journals for the general library. A highly satisfactory feature of the available departmental space was a large laboratory for graduate instruction. It has always seemed to me important that graduate students be located in close contiguity, for they can learn more from one another than from their formal instructors. For the same reason I have continually encouraged the greatest possible diversification in the subject matter of departmental researches.

On arriving in New York I found, on the biochemical staff, colleagues who proved to be towers of strength in the instruction of medical students: Edgar G. Miller, Jr., an incomparable lecturer; G. L. Foster, indefatigable

in the teaching laboratory; and Maxwell Karshan, who throughout the years has taken charge of the course for dental students. This group was soon joined by Crawford Failey, a physical chemist interested in biochemical problems, and Oskar Wintersteiner, a former student of Pregl, who on coming to the United States had collaborated first with P. A. Levene and then with J. J. Abel. The department also had the privilege of close association with Michael Heidelberger who was located in the Department of Medicine; he was then working on the chemistry of bacterial polysaccharides and subsequently developed his fruitful theories on the quantitative relationships in immunochemical reactions.

In the early thirties the junior staff was joined by Robert M. Herbst and Marianne Goettsch. Herbst, who had recently graduated from Yale, was primarily an organic chemist; after some years with us he left to go into industrial research but soon returned to academic life. Miss Goettsch, on the other hand, was an expert in nutritional biochemistry; after completing her formal training with us she ably represented her specialty in the instruction of our medical students. In 1942 she transferred to the Department of Biochemistry of the School of Tropical Medicine in San Juan, then affiliated with Columbia University, where she is now a professor in the University of Puerto Rico. Subsequent additions to our teaching staff have largely been recruited from the roll of graduates of Columbia University; these include David Rittenberg, now the head of the department, David Shemin, David Sprinson, and Stephen Zamenhof.

Among the many benefits which accrued to Columbia University from the racial policy adopted by the Germans under the Third Reich was the arrival in our laboratory of various European-trained biochemists, notably Erwin Chargaff, Zacharias Dische, Karl Meyer, Rudolf Schoenheimer, and Heinrich Waelsch. Erwin Brand, who joined our group during the same period, reached this country somewhat earlier. The scientific achievements subsequently made by these men are so well known that their enumeration is unnecessary.

Visiting biochemists or organic chemists who spent a year or more in the laboratory and thereby contributed to the stimulation and informal training of our students included Sune Bergström, R. J. Block, W. J. Darby, R. Gordon Gould, Max Huffman, R. C. Lewis, Gwei Djen Lu, M. S. Newman, E. S. West, W. W. Westerfeld, and R. R. Williams. Among others, some who worked in different departments but held academic titles in ours and took part in the instruction of medical or graduate students were L. S. Dietrich, Samuel Graff, Seymour Lieberman, Irving London, David Nachmansohn, Warren Sperry, and Irwin B. Wilson.

The circumstances under which Schoenheimer's most notable associate came into biochemistry may be of interest. When Harold Urey had prepared a sufficient quantity of heavy water to release some of it for work in labora-

tories other than his own, he secured from the Rockefeller Foundation funds for its exploitation in biological fields, and our department was one of the beneficiaries. With a supply of deuterium hydroxide came the opportunity to take in one of his assistants versed in the appropriate techniques. When David Rittenberg joined the laboratory in that capacity he was invited to circulate among the members of the group with a view to establishing a profitable collaboration. From his discussions with Schoenheimer came the idea of tracing the metabolic fate of a fat labeled with heavy hydrogen. The result of the very first experiment, which indicated that ingested fats were immediately deposited unchanged in the tissues, gave all of us an unforgettable thrill. It was not long before the novel concept of the dynamic state of body constituents had developed from the ensuing studies, and this was soon confirmed, in the area of protein metabolism, by experiments in which heavy nitrogen was employed as a tracer. The preparation of the labeled compounds, in which isotopic hydrogen and nitrogen were the limiting factors, involved many technical considerations unnecessary in the practice of classical organic chemistry.

The gratification afforded to the head of a department through the researches of his associates, great as it is, is equalled and perhaps surpassed by that derived from the success of his students. In both respects I have much cause for thankfulness. Of the 94 students who received their doctoral degrees in biochemistry from Columbia University during the period 1931 to 1956, many have made their mark as investigators and several are departmental chairmen in other universities. I cannot forgo the satisfaction of listing the names of those who have attained the national recognition implicit in election to the American Society of Biological Chemists or one of the other organizations which constitute the Federation of American Societies for Experimental Biology. These are Anthony A. Albanese (1940), Marjorie Anchel (1939), Herbert S. Anker (1945), Fred W. Barnes (1944), Aaron Bendich (1946), Konrad Bloch (1939), Ernest Borek (1939), George E. Boxer (1945), Seymour S. Cohen (1941), Thomas B. Coolidge (1938), James D. Dutcher (1940), David Elson (1952), Lewis L. Engel (1936), Bernard F. Erlanger (1951), Earl A. Evans (1937), Martin Flavin (1951), Joseph S. Fruton (1934), Marianne Goettsch (1931), Samuel Gurin (1934), C. H. Werner Hirs (1949), Henry D. Hoberman (1943), Elvin A. Kabat (1938), Alfred Linker (1954), Boris Magasanik (1948), Paul H. Maurer (1950), Manfred M. Mayer (1946), Abraham Mazur (1939), John W. Palmer (1933), William H. Pearlman (1940), Sarah Ratner (1937), Marianna Richards (1938; now Mrs. Max Bovarnick), Richard W. Schayer (1949), David Shemin (1939), David B. Sprinson (1946), William H. Stein (1939); DeWitt Stetten (1940), Marjorie R. Stetten (1945), Herbert E. Stokinger (1938), Gilbert C. H. Stone (1933), Paul K. Stumpf (1946), Norman Weissman (1941), and Stephen Zamenhof (1950). The figures in parentheses indi-

cate the official years of graduation. In addition to the above, two of our graduates, not enrolled in the Federation, are now leaders of productive research groups: Max Bovarnick (1939) and Samuel Graff (1932).

Admission of applicants to graduate studies in biochemistry at Columbia was, during my time there, always based on personal interview rather than on college grades. In these interviews, which frequently lasted an hour or more, an attempt was made to evaluate not only knowledge of and laboratory experience in the various branches of pure chemistry, but the way in which a candidate was able to coordinate such background information as he had acquired in college. After admission, prospective graduate students were frequently required to defer their entrance into the elementary course in biochemistry while they broadened their acquaintance with organic and physical chemistry, both theoretical and practical, at the graduate level. They also were often directed to take courses in biology as preparation for the study of human physiology, mandatory in the program. Such requirements were as a rule far from welcome to students eager to get on with their life-work, but their value usually became apparent to students in the later stages of their training, and was often frankly acknowledged.

It now seems appropriate to outline some of the ways in which organic chemistry has contributed to the development of biochemistry during the past half century. In my opinion two of the most influential factors have been of an essentially practical nature. The first was the elaboration by Pregl of methods of microanalysis and the manipulation of organic compounds on a milligram scale. The second is the more recent addition of chromatographic and countercurrent distribution procedures to the armamentarium of the biochemist. These modern techniques have made possible the emergence of physiological chemistry from a subject which, as admitted by Halliburton in 1904, was formerly regarded by chemists with scarcely veiled contempt, into an exact science. Without the help of these procedures, many more years would undoubtedly have elapsed between the recognition of the existence of enzymes, hormones and vitamins, and the present-day accurate knowledge of their chemical nature.

The exponential growth of biochemical knowledge is also largely ascribable to the perfection of methods for the isolation and synthesis of organic compounds of natural origin, since exact information concerning the chemical constitution of these compounds is essential for the elucidation of the chemical reactions which underlie vital processes.

I should like to illustrate these generalities by specific reference to some of the contributions, made in various laboratories during the past 50 years by organic chemists, or by biochemists with the aid of the methods of organic chemistry, which have particularly impressed me by reason of their practical utility, their technical elegance, or their influence in the expansion of biochemical concepts.

The part played by organic chemistry in the acquisition of knowledge of the chemistry of vitamins has of course been decisive; most of the work in this field, however, has been along well-recognized lines which added little of fundamental novelty to theoretical chemistry. Only exceptional cases will, therefore, be discussed here.

An early instance is that of the chemical changes induced by irradiation of certain steroids. The discovery by Hess in 1925 that cholesterol prepared in the usual manner develops antirachitic potency when exposed to ultraviolet light stimulated Windaus to survey the photochemical sensitivity of a wide variety of sterols, of which ergosterol was found to yield by far the highest degree of physiological activity. Careful study of the isomerization induced by light disclosed the successive migration of double bonds which culminated in the opening of Ring B, a result that could not have been foreseen. Calciferol, the antirachitic compound derived from ergosterol, was crystallized in 1931 by Bourdillon and by Windaus, working independently, but was found to differ from natural vitamin D in being less effective, per rat unit, towards avian rickets. This discrepancy was not observed, however, with the corresponding product from 7-dehydrocholesterol, crystallized in 1936 by Windaus and simultaneously isolated from tunny liver oil by Brockmann.

While these impressive researches were proceeding, investigations were being carried out by Kuhn and by Karrer on the constitution of riboflavin and its irradiation products. Here, again, the photochemically induced cleavage of the ribityl group, at position 1 in acid solution and position 2 in alkaline solution, could not have been predicted on the basis of existing knowledge. On the other hand, the chemistry of thiamine, the constitution of which was also unraveled during the middle thirties, offered little fundamentally new information. Pyrimidines and thiazoles were familiar classes of organic compound, though the latter had not previously been encountered in natural products. Rupture of the linkage between the two rings by oxidation was accomplished in 1934 by Windaus, but the concomitant degradation of the sulfur-containing constituent hindered the recognition of its exact chemical nature. In the following year, however, R. R. Williams discovered that this portion of the molecule could be split off essentially unchanged by the action of bisulfite, and this observation facilitated the determination of the constitution of the vitamin and its ensuing synthesis.

An outstanding example of skillful and imaginative application of organic chemical methods by a small group was afforded in 1941-1943 by du Vigneaud's work on the isolation, structure, and synthesis of biotin. This gem of research forms a striking contrast, relative to organization, with the recently completed determination of the constitution of Vitamin B<sub>12</sub>, a task which ultimately enlisted the efforts of a galaxy of experts in many fields and in various laboratories. A glance at the structural formula shown on



page 400 of the *Annual Review of Biochemistry* for 1956 should give justification for the opinion that here we have the most magnificent of all the achievements in the field of the chemistry of natural products.

In recent years the structure of antibiotic compounds of microbiological origin has attracted much attention from organic chemists. Reference will be made below to antibiotics of polypeptide nature; many, however, have proved to be compounds of smaller molecular dimensions, and some of these have been shown to contain chemical groupings previously encountered only in synthetic products. Penicillin contains thiazolidine and  $\beta$ -lactam rings and also an amino acid group with D configuration; nemotin is a lactone of a straight-chain hydroxy acid containing one allenic and two conjugated acetylenic groups; agrocybin is the amide of a triacetylenic hydroxy acid; chloromycetin (chloramphenicol) contains a *p*-nitrophenyl and a dichloroacetyl group; azaserine is an ester of diazoacetic acid; elaiomycin is an aliphatic azoxy compound. A compound which is not strictly an antibiotic but inhibits a sarcoma and was isolated from a streptococcal culture has turned out to be 6-diazo-5-oxo-L-norleucine. The therapeutically important antibiotics terramycin and aureomycin consist of highly substituted, partially hydrogenated derivatives of 2,3-benzanthracene. Streptomycin, a compound even more complicated in structure, contains three glycosidically linked units, two of which are of strikingly unusual character. The determination of the structures of these three "mycins" required intense labor by various large teams, mainly in industrial research laboratories, and represents organic chemical skill of the highest order.

As stated above, massive frontal attacks have not always been necessary for the solution of important problems in organic biochemistry. An example of fruitful research by a relatively small group is afforded by the isolation, identification and synthesis, in K. P. Link's laboratory, of the hemorrhagic agent in spoiled sweet clover hay. This achievement not only shed light on a matter of grave import to veterinary medicine but furnished an early example of the principle of biochemical antagonism, subsequently elaborated by Woolley. A parallel instance is the recognition of the chemical nature of the toxic factor produced in flour by the action of nitrogen trichloride. This product, the sulfoximine of methionine, represents a previously unknown type of compound which has widened the horizon of the organic chemist.

The chemistry of porphyrins exemplifies the development of an initially rather restricted field in a few laboratories. In this area the name of Hans Fischer stood supreme for many years; on the solid foundation constructed by him, Shemin has recently based a masterly explanation of the mechanism of the biosynthesis of heme from simple metabolites.

The chemistry of the steroid hormones, painstakingly elaborated by many groups during the past thirty years, beautifully exemplifies the con-

tributions of organic chemistry to biology and medicine. The constitution of the ovarian hormone estrone, isolated in crystalline form by Doisy and by Butenandt in 1929, was established in 1932 by Butenandt, who shortly thereafter isolated a male sex hormone and determined its constitution, confirmed two years later by Ruzicka. The analogy of the structures of the sex hormones to those just previously accepted for cholesterol and the bile acids suggested the possibility of a biochemical relationship; this was established in 1945 by Bloch's demonstration of the conversion in a human subject of deuterated cholesterol into deuteriopregnandiol. Absolute confirmation, according to the strict canons of organic chemistry, of the constitution of a sex hormone was supplied by Bachmann's total synthesis of equilenin in 1940, and by Johnson's synthesis of estrone in 1952.

The isolation of the hormones of the adrenal cortex offered even greater technical difficulties because of their large number and chemical similarities, but these were ultimately overcome by the work of independent groups led by Kendall, Reichstein, and Wintersteiner. Largely as a result of the chemical study of the sex hormones, the determination of the constitution of the cortical steroids proved less formidable. The synthesis of cortisone, though rendered difficult by reason of the unusual chemical characteristics of the hydroxyl group at position 11, was effected in 1952 by Sarett and his associates in the Merck laboratories. The constitution of aldosterone, which displays several unique chemical features, was determined in 1954 in a collaborative effort by Reichstein and a British group, the work being carried out with consummate skill on a total of 57 mg. of material. Its synthesis, by methods resembling those employed by Sarett, was completed by Wettstein in the following year.

The isolation of amino acids from protein hydrolysates and from other natural sources has required not only great skill in organic chemical manipulation, especially when previously unknown members of the class were involved, but an extensive knowledge of organic reactions in connection with their quantitative estimation and the determination of their constitution by degradative and synthetic procedures. Outstanding instances are the discovery of tryptophan by Hopkins & Cole; the isolation of thyroxine by Kendall and the demonstration of its constitution by Harington; the isolation of methionine by Mueller, its synthesis by Barger, and the demonstration of its biochemical relationship to cystine by du Vigneaud and others; the discovery of djenkolic acid by Van Veen and its synthesis by du Vigneaud; the isolation of canavanine by Kitagawa; the isolation of hydroxylysine by Van Slyke and its synthesis by Sheehan; the discovery of threonine by Rose and its synthesis by Carter; and the demonstration by Gross & Pitt-Rivers of the presence of tri-iodothyronine in plasma.

The quantitative separation of amino acids, essential for the establishment of a sound basis for an approach to the urgent problem of protein con-



stitution, has enlisted the attention of many skilled organic chemists subsequent to the early attempts by Emil Fischer. Help towards such efforts was afforded by the ingenious gasometric methods devised by Van Slyke, by Dakin's procedure for partial separation by extraction with butanol, and by Bergmann's development of the use of specific precipitants. Real progress, however, became possible only after the aid of specific microbiological mutants had been enlisted by Beadle and developed by Tatum & Bonner in 1944. However, the use of these was soon largely abandoned in favor of chromatographic procedures, first exploited by Martin & Synge and elaborated by Stein & Moore, Sanger, and others.

For many years the class of peptides was represented almost exclusively by synthetic compounds. The preparative procedures of Emil Fischer, which culminated in the production of an octadecapeptide, were rendered far more versatile by the introduction in 1932 of Max Bergmann's carbobenzyloxy method. No one but a keen organic chemist, alive to the possibilities of hydrogenolysis, would have conceived the idea of taking advantage of the lability of the benzyl linkage. Improved methods for the formation of peptides from optically active amino acids soon followed as a result of Bergmann's work. As is usual in such cases, most of these were developed by application of reactions previously discovered by others in purely organic chemical studies, such as the use of azides and mixed anhydrides in place of the classical acid halides, and of the condensing ability of highly reactive compounds like tetraethyl pyrophosphate. Another example is the application by Sheehan of Khorana's ingenious use of carbodi-imides, whereby not only peptides but  $\beta$ -lactams can be produced in either organic solvents or aqueous media. A major achievement by this procedure is Sheehan's practical synthesis of penicillins, a goal which the concerted work of many laboratories in this country and Britain failed to reach during the second World War.

With the exception of carnosine and anserine, which are not typical peptides, no naturally occurring compound of this class was known prior to the discovery of glutathione by Hopkins in 1921. The constitution of this tripeptide proved surprisingly difficult to solve, and was not rigorously established until 1935, when its synthesis was accomplished by Harington on the basis of evidence contributed independently by Hopkins, Hunter, Kendall, and Nicolet.

For some years thereafter, little attention was devoted to peptides from natural sources, until in 1943 Dubos & Hotchkiss prepared in crystalline form the antibiotic polypeptides gramicidin and tyrocidin, produced by certain bacilli. This discovery was followed by the isolation, in various laboratories, of several other products of similar character. It is interesting, and perhaps significant, that these antibiotics yield on hydrolysis some of their constituent amino acids in their D form. In several instances their constitu-

tions have been established; tyrocidine, for instance, was shown by Craig to be a cyclic decapeptide in which two of the members of the ring are D-phenylalanyl groups.

The methods by which the sequence of the components of such peptides was determined command the intense admiration of the organic chemist. They involve the isolation of multitudes of simpler peptides from partial hydrolysates, by Craig's countercurrent distribution or by chromatography, and the identification of their terminal amino groups by Sanger's procedure involving labeling with dinitrofluorobenzene. Impressive, also, is the intellectual feat of assembling the scraps of information thus obtained into a picture from which the sequential constitution can be deduced.

Attempts to devise more direct and less laborious procedures have not been lacking. The most promising of these seems to be that of a stepwise degradation, proposed in 1949 by Edman, based upon the rapidity with which phenylthiohydantoins (or phenylthiohydantoins) are detached by acid from the corresponding ureides, with production of the next lower member of the peptide chain. Unfortunately, simultaneous hydrolysis of peptide bonds occurs to an extent which, though negligible in the first step, cumulatively complicates the isolation of significant products.

Undoubtedly the outstanding success in the field of polypeptides is that attained with the hormones of the posterior pituitary. After years of relatively sterile effort by various groups, the isolation in crystalline form of pure oxytocin and of two varieties of vasopressin was successfully accomplished in 1953 by du Vigneaud and by Fromageot, working simultaneously and independently. Both groups then determined the structures of their products, with entire concordance. In an astonishingly short time the Cornell group then confirmed the constitution of all three hormones by synthesis, an achievement of first rank in the annals of organic biochemistry. In this work, extensive use was made of the recently developed method of peptide formation by means of tetraethyl pyrophosphite. A fortunate circumstance was the fact, previously established by du Vigneaud, that in these closely related cyclopolypeptides the disulfide bridges which close the ring can be restored unchanged after reduction.

Considerable progress has also been made during the past few years in the determination of the structure of bacitracin A, the principal component of a mixture of antibiotic polypeptides elaborated by a rare bacillus which had been isolated from a wound in a patient named Tracy. In addition to yielding several D-amino acids on hydrolysis it exhibits other unusual structural features, the most challenging of which is the presence of a masked sulfhydryl group, uncovered when the antibiotic activity is destroyed by mild acid hydrolysis. Craig in the United States and Abraham in England, who have carried out most of the work on this cyclopolypeptide, have adduced evidence that the sulfur atom is present in a thiazoline ring, apparently

formed between D-isoleucine and L-cysteine residues. Abraham and his group have recently shown that the  $\epsilon$ -amino group of lysine is involved in the closure of a ring containing six amino acid residues and that its  $\alpha$ -amino group forms the point of attachment of a side-chain.

It is not surprising that polypeptides should have been subjected to elaborate structural studies, for they furnish comparatively simple models for attack on the constitution of proteins. A most impressive exploration in this vast and intricate area has recently been completed by Sanger in the case of insulin.

The history of insulin chemistry presents many episodes of special interest to the organic chemist. The recognition of its protein nature by Dudley in 1923 was confirmed by Abel after he had crystallized it in 1926, the same year, incidentally, as that in which the first crystalline enzyme, urease, was prepared by Sumner. In 1927, du Vigneaud, working in Abel's laboratory, demonstrated the presence of labile disulfide groups in insulin and showed that on reduction of these linkages the physiological activity thereby lost was not restored on reoxidation (an effect strikingly contrasted by his later findings with oxytocin). Intensive search in various laboratories for a prosthetic group proved fruitless, and subsequent quantitative studies confirmed the view that insulin contains nothing but amino acid groups. Molecular weight determinations by physical methods suggested that the protein consists of associable units of magnitude about 12,000. In 1952 Craig tested the validity of this figure in an ingenious approach, made possible by a combination of Sanger's procedure, developed in 1945, for the labeling of amino groups in insulin with the dinitrophenyl radical and his own method for the separation of mixtures by means of countercurrent distribution in systems of incompletely miscible liquids. In principle, the dinitrophenyl content of the individual fraction containing only one such added group should constitute a measure of the molecular weight. This method was first rehearsed with the crystalline antibiotic polypeptides gramicidin S and polypeptin, and gave results consistent with data secured by quantitative amino acid analysis. When applied to partially dinitrophenylated insulin it yielded a molecular weight value of 6500. A similar figure was obtained from a fraction consisting of disubstitution products.

In his earliest (1945) work on dinitrophenylated insulin, Sanger showed that the hydrolysate from a fully substituted product contained only three labeled amino acids, namely glycine, phenylalanine, and lysine (substituted in the  $\epsilon$ -position), in amounts corresponding to two of each on the basis of a molecular weight of 12,000. Four years later, Sanger published the important observation that insulin is split into two polypeptides when the disulfide groups are oxidized to sulfonic acid groups by performic acid. One of these, Fraction A, proved to contain glycine in the amino terminal position; on the basis of its apparent molecular weight, about 2500, only one glycine

residue was present. The other components, all of which were quantitatively estimated, included four cysteic acid groups, at least one of the sulfur atoms of which must have been involved in the original linkage of the two peptide chains. The other polypeptide, Fraction B, was found to have a molecular weight about twice as great and to contain phenylalanine in the N-terminal position.

Sanger and his group then heroically undertook the task of determining the amino acid sequences in the two polypeptides by subjecting them to partial hydrolysis by means of acids and proteolytic enzymes, separating and identifying the resulting simpler peptides, and fitting the results together as in a jigsaw puzzle. In a phenomenally short time they completely solved this problem insofar as the sequence of constituent amino acids was concerned. The position of the amide groups was then cleared up by means of determinations of the ionophoretic mobilities and amide contents of peptides from enzymic hydrolysates of the two fractions from the oxidized insulin. Establishment of the constitution of the hormone itself, which involved determination of the position of the disulfide linkages, was complicated by disulfide interchanges during hydrolysis. A study of this reaction with simple models showed that in neutral or alkaline solution it is catalyzed by thiols and inhibited by thiol-binding agents, whereas in acid solution it is inhibited by thiols. With this information, suitable conditions could be selected for the partial hydrolysis of insulin; with the aid of ionophoretic separation of the resulting peptides the complete structure was determined. Chain A contains 21, chain B 30, amino acids in sequence, the two chains being doubly connected by disulfide links at points 6 and 20 in chain A to points 7 and 19, respectively, in chain B. These chains are actually cyclic in form owing to the presence of internal disulfide groups, which in the case of chain A is of the same size as that in oxytocin. Finally, Sanger and his colleagues have shown that insulins from cattle, swine, and sheep differ in amino acid composition at points 8, 9, and 10, but that the constitution of chain B is the same in the three species. The molecular weights of these structures conform with the findings of Craig.

With these remarkable achievements, a long-standing primary goal of organic biochemistry—the constitution of a protein—has now been reached. As much admirable work, along analogous lines, is being conducted in other laboratories with other proteins, a new era in protein chemistry has undoubtedly been entered.

The topics above outlined represent merely a few examples, arbitrarily selected, of the achievements of organic chemistry in the solution of problems posed by biochemistry. No reference has been made to the many notable contributions, such as the demonstration by Westheimer & Vennesland of the stereospecificity of DPN-linked dehydrogenation reactions, which have been effected with the aid of techniques peculiar to the biochemical

laboratory. It is surely obvious that the scope of research by the biochemist is greatly widened by a command of the theory and practice of organic chemistry. A significant proportion of the work signalized in these Annual Reviews has emanated from biochemical laboratories directed by scientists with extensive experience in the organic field.

Similar considerations apply to the increasingly important role played by physical chemistry in biochemical research. Biochemists in charge of the training of graduate students would therefore, in my earnest opinion, do well to discourage too early specialization and to insist on the acquisition of a thorough grounding in the fundamental branches of chemistry prior to embarkation on the intensive study of biochemistry.

# CHEMISTRY OF THE CARBOHYDRATES<sup>1</sup>

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As has been the custom in recent issues of the Annual Review this chapter deals with a few topics selected from the broadly expanding field of carbohydrate chemistry.

## SOME CONSEQUENCES OF THE CONFORMATION OF CARBOHYDRATE MOLECULES

*Theoretical and physical approaches.*—As the awesome diversity of reactivities exhibited by configurational isomers continues to unfold, the need for generalizing concepts becomes more urgent. The facts that conformational analysis, together with information on reaction mechanisms, may contribute to the formulation of useful generalizations, and that significant contributions have been made since the review by Bourne & Stephens (1) justify consideration of this topic again, only two years later.

Biochemists have long realized that the shapes (conformation) of carbohydrate molecules would be a crucial factor in the understanding of their enzymatic and immunological reactions. That shape would also play a major role in determining physical and chemical properties was recognized in two recent symposia dealing with reaction mechanisms and structure. The Chemical Society symposium is reported briefly in its Proceedings (2), and abstracts of the symposium held by the American Chemical Society are available (3).

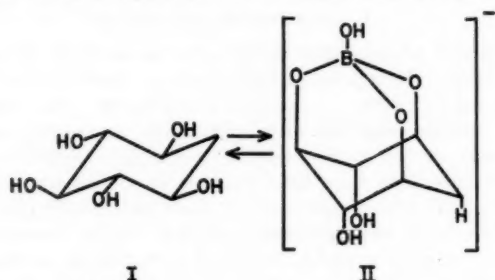
In a contribution of unusual interest, Whiffen (4) has attacked the problem of the relationship between molecular shape and optical rotation with considerable success. His rotational parameters apply only to substances in a chair conformation. It is interesting to note that the newly calculated values accommodate many configurations including the anomeric forms of mannose quite well, but fail with the altroses, lyxoses, and their glycosides. These failures are considered to be in accord with mounting evidence that the latter substances do not have a stable chair conformation. The agreement between calculated and observed values for the initial rotation of  $\beta$ -D-ribose lends support to the idea that crystalline ribose is a pyranose.

When one considers the benefits which have arisen in the past from the application of Hudson's rules of isorotation to problems of structure and purity, it may confidently be predicted that this new tool will be of great value to the carbohydrate chemist. It appears that the author is under an obligation to extend the method to include the methyl ethers and, especially,

<sup>1</sup> The survey of the literature pertaining to this review was completed in October, 1957.

the acetates. This reviewer has misgivings on the use of the calculated and observed rotations of conformationally unstable configurations to calculate percentages in each of the chair forms. As will appear later, there are conformations other than the chair forms which demand consideration in these instances.

In a very brief article Angyal & McHugh (5) have reported a partial solution of what has appeared to be a most difficult problem in the field of conformational analysis; the quantitative evaluation of nonbonded interactions. Working with the quercitols and inositols, these authors have determined equilibrium constants for the formation of tridentate (bird-cage) borate complexes of the type shown for *scyllo*quercitol, I and II. The free



energy change due to complexing,  $\Delta F_B$ , was found to be  $-2.5 \pm 0.3$  kcal./mol. and the nonbonded interactions were calculated to be as shown in Table I.

TABLE I  
NONBONDED INTERACTIONS IN THE CYCLOHEXITOL SERIES\*

Interaction†	kcal./mol.
O <sub>e</sub> :O <sub>e</sub> or O <sub>e</sub> (on adjacent ring atoms)	$0.35 \pm 0.07$
O <sub>a</sub> :H <sub>a</sub> (on 1, 3 ring atoms)	$0.45 \pm 0.05$
O <sub>a</sub> :O <sub>e</sub> (on 1, 3 ring atoms)	$1.9 \pm 0.1$

\* From Angyal & McHugh (5).

† The subscripts *a* and *e* signify axial and equatorial substitution on a ring atom, respectively.

The numerical values for the interactions apply only to chair forms. Although these values were obtained with cyclohexitols they will certainly be useful approximations for calculations in the pentose series of pyranoses; extension of the interactions to include the carbinol group would allow general application to the hexopyranoses. The authors have already applied their group interaction values to hexopyranoses in a special sense to arrive at an ex-



planation for the formation of 1,6-anhydrohexopyranoses under acid catalysis.

Scyllitol possesses the inositol configuration having a chair conformation permitting all six hydroxyl groups in the equatorial orientation (the C1, 1C convention is meaningless for such substances). Using the values given above one may calculate the free energy change for tridentate borate complexing on scyllitol,  $\Delta F_s$ , to be

$$\Delta F_B + 3(O_s:O_s) - 6(O_s:O_s) = 1.1.$$

On the basis of its calculated positive  $\Delta F_s$  value, scyllitol would not be expected to form a tridentate borate complex. Nakajima *et al.* (6) have discussed the conformations to be expected of the konduritol (tetrahydroxycyclohexanes).

Recent reviews on the conformation of six-membered rings (7, 8, 9) have overlooked an important theoretical paper by Hazebroek & Oosterhoff (10) dealing with the cyclohexane ring. These authors offer mathematical proof that there are an infinite number of possible conformations for the six-membered ring which are devoid of angular strain. By presuming that the geometry of the tetrahydropyran ring will resemble that of the reduced six-carbon ring, the way is opened for an advance in the theoretical treatment of pyranose conformations. To be readily depictable on a two dimensional surface a six-membered ring must have four or more atoms in a plane. Of the six general depictable conformations shown in Figure 1 the upper two are improbable since the ring-forming bonds must be highly strained from the tetrahedral angle. Although there is no necessary relationship between depictability and stability, depictability is a convenience in the communication of ideas regarding ring shapes.

Following Hazebroek & Oosterhoff, a strainless cyclohexane ring may exist in a rigid chair form or a flexible form which encompasses the boat and skewed (stretched) forms and an infinite number of shapes intermediate

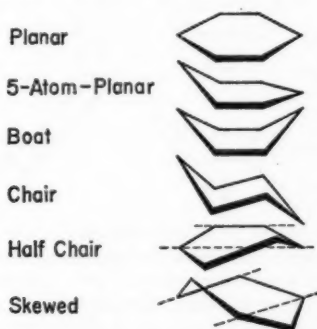


FIG. 1. The six depictable general conformations for the six-membered ring.



between the latter. Translated into the realities of the pyranose system, where each conformation yields a unique structure, there will be two different rigid chair forms and an infinite number of flexible forms which are describable as belonging to a cycle containing six specific boat forms. Figure 2a defines the specific chair and boat conformations and symbols which have been proposed representing each form (11, 12, 13). The interrelationships among the flexible forms are illustrated in Figure 2b which shows the order of interconvertibility of the six boat forms (14). Midway between each pair of boat forms will be a depictable skewed conformation; however, all of the shapes in the flexible cycle, whether depictable or not, are free from angular strain. If a particular carbohydrate configuration, because of non-bonded interactions, does not possess a stable chair conformation it may find a stable shape somewhere in the flexible cycle. Examination of molecular models makes such a possibility particularly attractive for the lyxose and altrose configurations and for mucinositol. The behavior of mucinositol upon periodate oxidation was not in line with that expected from a chair conformation for this substance (15). In discussing the formation of triisopropylidene derivatives of (-)-inositol and epiinositol, Angyal & Macdonald (16) were forced to postulate a highly distorted chair form. It is now recog-

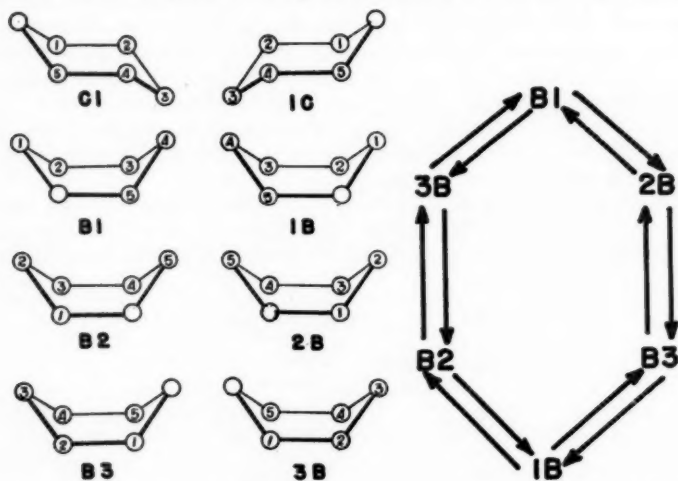


FIG. 2. (a) A definition of the symbols representing the two chair and six boat conformations for the pyranose ring. The numbering of the atoms is an integral part of the definition and follows the conventions of carbohydrate nomenclature. The heavy lines represent the side of the three dimensional figure nearest the observer and the open circles, the ring oxygen atoms. (b) A diagrammatic representation of the possible pathways for the interconversion of boat forms in the strainless flexible cycle. A skewed conformation occurs midway between each two adjacent boat forms in this cycle.

nized that there are skewed conformations which might very easily accommodate the three isopropylidene rings in each of these substances.

Lemieux *et al.* (17) have investigated the nuclear magnetic resonance (NMR) spectra of acetylated carbohydrates; Isbell *et al.*, (13) the infrared spectra of acetylated carbohydrates; Kelly, the refractive index of methylated glycosides (18), and in all three investigations features of conformational significance were detected. The NMR spectra distinguish between protons of axial and equatorial acetyl groups or between axial and equatorial hydrogens situated on the anomeric carbon atom. The infrared absorption in the regions 8.63 and 8.87  $\mu$  appears to be sensitive to the orientation of the acetyl groups on the anomeric carbon atom. Physical techniques such as these have an advantage over chemical procedures for studying ring shapes in that they do not alter that which is under investigation. Much can be learned by the extension of work with these techniques.

*Chemical approaches.*—Chemical approaches to conformational problems have been reported by a large number of workers. It is frequently the orientation of a group, whether axial or equatorial, or the relative position of two groups which determines the course or rate of a reaction. By interpreting chemical behavior with due allowance for probable ring conformations a great deal of progress can be made toward the generalization of carbohydrate reactions.

Richtmyer and co-workers have continued their investigation of the formation of 1,6-anhydrohexopyranoses (or 2,7-anhydroheptulopyranoses) under the influence of acid catalysis (19). In D-series hexoses and heptuloses this type of anhydride requires the 1C ring conformation, and in a general way the extent of formation of the anhydride is found to be proportional to the predicted stability of the 1C ring form. However, these workers have noted that an axial hydroxyl group at position 3 in the 1C form would produce a considerable strain in the anhydro structure so they have begun an investigation of the 3-deoxy sugars (20). In accordance with the idea of the relative importance of the substituents at position 3 they have observed that the tendency for anhydro formation by 3-deoxyarabinohexose is intermediate between that of altrose and mannose, and 3-deoxyribohexose is intermediate between that of allose and glucose. Mannoheptulopyranose (primary carbinol group equatorial in 1C form) produces distinctly more anhydride than does mannose under comparable conditions (21).

Bentley has studied the rate of oxidation of anomeric methyl glycosides by hypochlorite, finding that a substance with an equatorial glycosidic  $-\text{OCH}_3$  group is more rapidly attacked than one with the group axial (22). Extension of these studies to the disaccharides, maltose and cellobiose, has indicated that the oxygen atom linking the monosaccharide units is, in both cases, equatorial (23). This finding would require assignment of some conformation other than C1 for the nonreducing glucose unit of maltose. Shafizadeh & Thompson (24), in considering the factors influencing the rate of hydrolysis of pyranosides concluded that conformational instability, size and nature of the group on C5, and axial or equatorial orientation of the

glycosidic group were the more important factors. The  $\alpha$ -glycosides (axial) are generally more resistant to hydrolysis than the  $\beta$ -glycosides (equatorial), but inversion of this order was noted for the anomeric disaccharides, maltose and cellobiose. If Bentley's finding that the glucose units of maltose are equatorially linked is correct, this feature, plus the instability of the glucose unit in a nonchair conformation, might explain the relative rates of hydrolysis of maltose and cellobiose. It is noted that the equatorial linkage of the  $\alpha$ -glucopyranosyl units in maltose would be in agreement with a suggestion that the units of amylose are so linked (1, 25).

Khorana *et al.* (26) using the reagent dicyclohexylcarbodiimide to promote the formation of cyclic phosphates, have found that cyclic phosphates with five-membered rings form when the phosphate and an adjacent hydroxyl group bear the  $a:e$ , or  $e:e$  relationship, but not if the relationship is  $a:a$ . It was the failure of  $\alpha$ -D-mannose 1-phosphate, supposedly in the C1 conformation, to form a five-membered cyclic phosphate which led to the conclusion that the  $a:a$  relationship is unreactive. It is not fully understood at this time why the mannose phosphate was unable to undergo some conformational rearrangement which would bring the phosphate and hydroxyl group into an orientation favorable for the reaction to proceed. There are, among the flexible ring forms, several possible conformations which would appear to offer relatively little nonbonded interaction, and which would allow a close approach of the substituents at positions 1 and 2 of the  $\alpha$ -D-mannopyranose configuration.

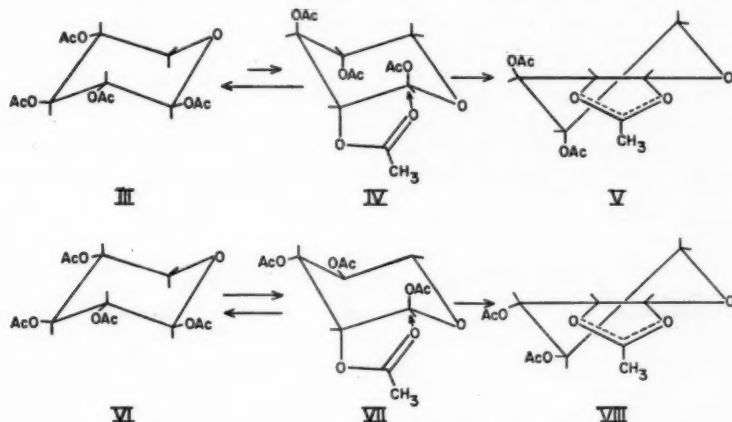
There is considerable evidence that equatorial hydroxyl groups may be esterified more readily than axial hydroxyl groups. Taking advantage of the expected preferential reaction of the equatorial groups, Aspinall & Zweifel (27) partially tosylated methyl 4,6-O-ethylidene- $\alpha$ -D-mannoside and 1,6-anhydro- $\beta$ -D-mannopyranose. These substances are believed to exist in the C1 and 1C forms, respectively. In these ring conformations the former substance would have an equatorial hydroxyl at position 3, the latter, at position 2; and these are the positions found to be selectively tosylated. Methylation of methyl 3-O-tosyl-4,6-ethylidene- $\alpha$ -D-mannoside followed by removal of the blocking groups provided an elegant synthesis of the difficultly accessible 2-O-methylmannose. Jeanloz & Jeanloz (28) and Bolliger & Prins (29) studied the partial esterification of methyl 4,6-O-benzylidene- $\alpha$ -D-glucoside, a substance which is expected to exist in the C1 conformation having both hydroxyl groups at positions 2 and 3 in the equatorial orientation. Monotosylation of this compound gave 80 to 85 per cent of the 2-tosyl derivative. In this case there are, also, strong directive influences. What these influences are and what part they may have played in determining selectivity in the mannoside series will have to be determined before it can be concluded that the results in the latter series were attributable largely to the equatorial-axial relationships.

Newth has discussed the conformational aspects involved in the formation of epoxides from various tosyl derivatives (30). He recognizes that the half-chair conformation can best account for the structure of epoxides.

Evidence that D-glucosamine derivatives exist in the same ring form as D-glucose, the C1 form, was obtained in studies on the migration of acetyl from the 3-O- to 2-N- position (31). It was argued that this migration would have taken place only if both substituents were equatorial.

Reeves & Blouin observed that the optical rotations of the methyl glycosides of  $\beta$ -altropyranose,  $\alpha$ - and  $\beta$ -galactopyranose,  $\beta$ -mannopyranose, and  $\beta$ -arabinopyranose are reversibly altered upon going from neutral to alkaline solution (14). They suggested that the change in rotation might be due to the tendency of ring hydroxyl groups to assume an equatorial orientation in strongly alkaline media. However, there are other glycosides, presumed to have axial hydroxyl groups, which are not alkali-sensitive. The shift in optical rotation under alkaline conditions is also exhibited by methyl  $\beta$ -maltoside, sucrose, and, to a pronounced extent, by amylose. It appears likely that the effect is related to conformational changes in some manner, but a completely satisfactory explanation has not yet been made.

Lemieux & Brice studied the rates of exchange of the acetoxy group at carbon one with carbon-14 labelled stannic trichloride acetate in a number of 1,2-*trans* sugar acetates (32). Among their findings was the fact that the 1-acetate of  $\beta$ -D-xylopyranose tetraacetate exchanges much more rapidly than that of  $\alpha$ -D-lyxopyranose tetraacetate. The initial and intermediate stages in this exchange reaction are depicted for D-xylose, III to V, and



L-lyxose, VI to VIII. The authors presume that the first step, the rearrangement to a reactive conformation, proceeds less readily for xylose (IV) than for lyxose (VII), but that the second step proceeds the more readily in the case of xylose. They reason that it is the configuration of the group at position 3 which has the overriding influence. The *trans* orientation of substituents at positions 2 and 3 in V allows a planar five-membered ring with resonance stabilization. In VIII the *cis* orientation of the substituents at

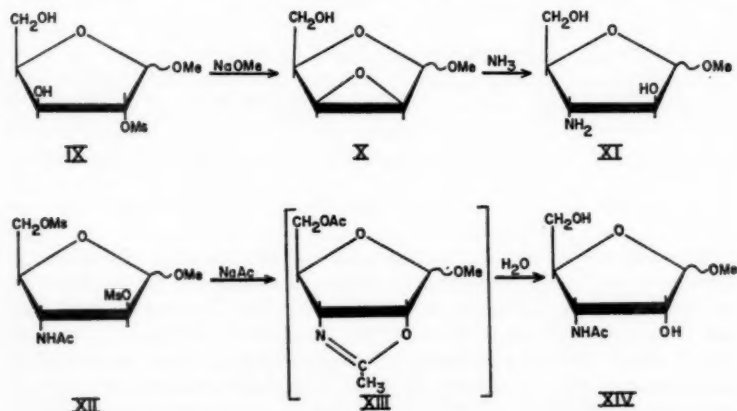
2 and 3 tends to deform the planarity of the five-membered ring with loss of resonance stabilization. These authors amply support their thesis that in order to rationalize carbohydrate reactivities one must consider not only the most stable conformation (the ground state), but also the stabilities of the transition ring forms.

Barton has described the construction of models which are particularly suitable for conformational analysis (33).

#### CONFIGURATIONAL TRANSFORMATIONS

The classical epimerization reaction was successfully employed by Prentice *et al.* to prepare 2,4,6-tri-O-methyl-D-mannose from the corresponding D-glucose derivative (34). The former substance was identical with a trimethylmannose obtained following methylation and hydrolysis of a glucomannan from *Puccinia graminis tritici*, an organism causing stem rust of wheat. Dilute barium hydroxide at 35° produced an approximately equimolar mixture of the two sugars, and 31 per cent of the desired substance was readily isolated by chromatography. Although a similar result was obtained thirty years ago with the tetramethyl sugars (35) this new work serves as a reminder that epimerization is still a useful tool and one which should be further investigated with other configurational isomers.

*Transformations involving sulfonyl esters.*—The alkaline hydrolysis of sulfonyl esters frequently involves configurational transformations at two asymmetric centers in a glycosidic molecule and this reaction has been used frequently to enter the altrose, idose, and talose series starting from the more available configurations. Briefly mentioned last year in these pages was a synthesis by Baker, Schaub & Williams of 3-amino-3-deoxy-D-ribose (36) which proceeded through all four D-pentose configurations in the order xylose→lyxose→arabinose→ribose, IX to XIV. The first two transformations, IX to XI, involved the removal of the sulfonyl group, in this case



methanesulfonyl (mesyl, Ms), with the formation of an epoxide ring, and the *trans* cleavage of the epoxide with ammonia. Anderson & Percival have also investigated this particular sequence of reactions (37). Situations in which a sulfonyl group is removed with or without Walden inversion have been discussed by Tipson (38). Conditions favorable for the formation of epoxide rings occur when a sulfonyl ester and a free hydroxyl group on an adjacent carbon atom have the *trans* orientation, or can easily achieve this orientation by conformational adjustment. Walden inversion then occurs at the carbon atom bearing the sulfonyl group. The epoxide may or may not be isolated. It, however, can undergo *trans* cleavage to reform the original configuration, or a new configuration in which two asymmetric centers have been inverted. Newth has investigated the saponification of *p*-toluenesulfonyl (tosyl) esters of 1,6-anhydro- $\beta$ -altropyranose, a substance having equatorial substituents at positions 2 and 3, axial at position 4 (30). Because of the anhydro ring it would be very difficult for this molecule to orient any adjacent substituents in the *trans* position. The 2-tosyl ester was highly resistant to alkaline hydrolysis, the 3-tosyl ester gave 1,6;3,4-dianhydro- $\beta$ -D-altropyranose, without inversion.

The removal of a sulfonyl group to form an oxazoline ring, XII $\rightarrow$ XIII, occurs with an acetamido group *trans* to the sulfonyl substituent. The carbon atom bearing the sulfonyl group undergoes Walden inversion, but the oxazoline ring, unlike the epoxide ring, may be opened with retention of the *cis* orientation of its substituents, XIII $\rightarrow$ XIV. A new 2-amino-2-deoxy sugar, D-gulosamine, has been isolated by Van Tamelin *et al.* from streptothricin (39), and synthesized by Tarasiejska & Jeanloz by a route similar to steps XII to XIV. The starting material in this case was methyl N-acetyl-3-O-mesyl- $\alpha$ -D-galactosaminide (40). The hexosamine prepared by Kuhn *et al.* (41) does not have the same initial rotation as gulosamine and, from its method of preparation, might be D-idosamine. D-Allosamine was also prepared, from a derivative of D-glucosamine, by means of the oxazoline reaction with inversion at position 3 (42).

The ribose configuration has been achieved by the hydrolysis of 2-O-mesyl-D-arabinose (43) and its 4-O-formyl ester (44). That this reaction proceeds through the formation of an epoxide involving position 1 was shown by Smith who used sodium methoxide to obtain a mixture of methyl pentosides consisting of methyl  $\beta$ -D-riboside and the anomeric forms of methyl D-arabinoside (44).

*Other transformations.*—Closely related to the sulfonyl esters are the sulfate esters which undergo alkaline hydrolysis to produce similar configurational transformations. Overend & Ricketts have prepared dextran sulfate and subjected it to alkaline, then acid hydrolysis isolating D-altrose and D-mannose in addition to D-glucose (45). In this instance the conversion from the glucose configuration must have taken place in the monosaccharide units of the 1,6-linked polysaccharide. If such a transformation could be carried out with the 1,4-linked polysaccharides some highly interesting modified celluloses and starches might result. Sulfated 1,6-anhydro-D-

glucopyranose also gave small yields of altrose and mannose after the two hydrolysis steps.

The Amadori rearrangement which produces N-substituted 1-amino-1-deoxy ketoses from N-glycosides has been the subject of continued investigation (46, 47). Micheel & Frowein have found that alkyl N-glucosides which hitherto have failed to yield crystalline products in the Amadori rearrangement do so in good yield if the 4,6-O-benzylidene derivatives of the N-glucosides are employed as the starting materials (48). Moffatt & Khorana have observed the D-xylose 5-phosphate is rapidly isomerized to xylulose 5-phosphate (D-threopentulose 5-phosphate) in neutral solution (49).

The enzymatic transformation of glucose to fructose by xylose isomerase (50), and the biosynthesis of L-gulonic acid from D-glucurone or D-glucose (51, 52) have been observed. Topper has reported some interesting observations upon the action of phosphoglucose isomerase and phosphomannose isomerase on fructose 6-phosphate (53). It was shown with the aid of deuterium labelling that these enzymes distinguish between the hydrogen atoms at carbon one of the substrate, forming either a *cis* or *trans* fructose enediol depending upon which enzyme is involved.

A series of reactions which has the over-all result of producing a configurational transformation is the synthesis of L-iduronic acid from the D-glucose configuration. L-Iduronic acid is of considerable interest to biochemists since its isolation from chondroitin sulfate B by Hoffman, Linker & Meyer (54). Its synthesis involves the cleavage, by periodate, of 1,2-O-isopropylidene-D-glucofuranose to give 5-aldo-1,2-O-isopropylidene-D-xylopentofuranose, a substance which undergoes interesting dimerization and condensation reactions (55). The cyanhydrin reaction upon the 5-aldo substance produces the L-*ido* and D-*gluco* configurations from which the corresponding uronic acids are eventually obtained (56). This synthesis has recently been carried out by Schaffer & Isbell with C<sup>14</sup> labelling at position six (57). Another entry into the L-idose series was reported by Dekker & Hashizume, who hydrolyzed 5,6-anhydro-1,2-O-isopropylidene-D-glucofuranose, obtaining, in addition to D-glucose, a 25 per cent yield 2,5-anhydro-L-idose (58).

Burma & Horecker obtained from *Lactobacillus pentosus* an enzyme which catalyzes the reversible transformation of the phosphates of L-erythropentulose (L-ribulose) and D-threopentulose (D-xylulose) (114).

#### OLIGOSACCHARIDES

Four years ago in these pages Whistler & McGilvray wrote, "The number of oligosaccharides theoretically producible is astronomical and many hundreds may be isolated within the next few years" (59). Fulfillment of this prediction has resulted in the necessity of restricting this topic, with a few exceptions, to the consideration of the di- and trisaccharides. Recent reviews of this subject include those by Blair & Pigman (60), and by Hassid



& Ballou in Pigman's new book (61). Frequent reference to the latter work will be made in order to reduce the length of this section.

One conscious deviation from recommended nomenclature (62) will consist of the omission of the —O— to designate glycosyl linkage through oxygen whenever the horizontal arrow is employed to indicate the position of linkage in oligosaccharides. Since the arrow is a symbol, it may as well serve the double duty of indicating the position of union and the fact that the union is through oxygen. After all, these substances would not be di-, tri-, and tetrasaccharides, etc., if the linkages were not through oxygen.

*The D-glucose saccharides.*—The non-reducing disaccharide trehalose,  $\alpha$ -D-glucopyranosyl  $\alpha$ -D-glucopyranoside, has been encountered by Howden & Kilby in unusually high concentrations, as much as 1.5 per cent, in the blood of desert locust nymphs at certain stages in their development (63). It has been found by Lederer and co-workers in a highly toxic lipid isolated from *Mycobacterium tuberculosis*. This toxic lipid, the so-called "cord factor," is identified as trehalose 6,6'-dimycolate (64, 65), mycolic acid being a high molecular weight, branched-chain, hydroxy acid. The finding of trehalose in the *M. tuberculosis* lipid recalls the earlier work of Anderson and associates who found this disaccharide, instead of glycerol, in the neutral fats from the same organism (66). Trehalose and its  $\alpha,\beta$  and  $\beta,\beta$  isomers have been synthesized (61, 67).

$\beta$ -D-Glucopyranosyl-(1 $\rightarrow$ 2)-D-glucose (sophorose), has been isolated from natural sources and synthesized (61). It has recently been found by Vis & Fletcher to be the disaccharide bound in glycosidic linkage in stevioside, the intensely sweet principle of *Stevia ribaudiana* Bertone (68).

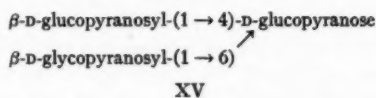
Nigerose,  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-D-glucose continues to be found in partial hydrolysates of starches (69) and glycogen (70) in amounts which indicate that the  $\alpha$ -1 $\rightarrow$ 3-linkage is an integral part of these structures. Pazur *et al.* have achieved synthesis of nigerose from maltose and glucose using an enzyme from *Asperigillus oryzae* (71). The two reducing trisaccharides with alternating  $\alpha$ -1 $\rightarrow$ 3- and  $\alpha$ -1 $\rightarrow$ 4-linkages have been found, along with nigerose, among the partial hydrolysis products of the polysaccharide, nigeran (72).  $\beta$ -D-Glucopyranosyl-(1 $\rightarrow$ 3)-D-glucose (laminaribiose), the  $\beta$ -linked isomer of nigerose, has been isolated from natural sources, and has been synthesized (61).

Recent work with maltose,  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose, concerns the conformation of the nonreducing glucosyl moiety (23). The isolation and characterization of homologous members of the  $\alpha$ -1 $\rightarrow$ 4 reducing saccharides through the heptasaccharide has been reported (73). The transfer of glucosyl (or maltosyl) residues by potato D enzyme from maltodextrins to methyl  $\alpha$ -D-glucopyranoside has resulted in the preparation by Peat, Whelan & Jones of the methyl  $\alpha$ -glycosides of maltose, maltotriose, and maltotetrose (74). It is interesting that this appears to be the first preparation of methyl  $\alpha$ -maltoside,  $[\alpha]_D^{20} = 180^\circ$ . The calculated molecular rotation



of this substance minus that of the  $\beta$ -anomer, 64,080 - 29,900 (75), gives a 2A value of 34,180, in fair agreement with the value 36,600 calculated for the  $\alpha$ - and  $\beta$ -anomers of methyl glucopyranoside (76). From this one might suppose that the methyl glucoside portion of the methyl maltosides exist in the normal chair conformation. Panose,  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose, has been obtained among the enzymatic digestion products of starch (77, 78).

In the  $\beta$ -1 $\rightarrow$ 4-linked series Klemer has prepared the 4-6-O-ethylidene derivative of cellobiose (substituent on the nonreducing glucose residue) and employed this to synthesize derivatives of XV, a branched-chain trisaccharide (79).



This trisaccharide, contains both the cellobiose and gentiobiose linkages. Lemieux, Thorn & Bauer have found cellobiose to be combined in  $\beta$ -glycosidic linkage in the ustilagic acids A and B. Although cellobiose represents the basic unit of cellulose this is the first instance of its being found as a true glycoside in biological material (80).

$\beta$ -D-Glucopyranosyl-(1 $\rightarrow$ 5)-D-glucose has been isolated by Sowden & Spriggs from hydrol where it probably occurs as an acid reversion product of glucose (81). Apparently, the  $\alpha$ -linked isomer of this disaccharide is unknown.

Isomaltose,  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-glucose, and the homologous  $\alpha$ -1 $\rightarrow$ 6-linked tri- and tetrasaccharides were obtained by the enzymatic hydrolysis of dextran by Jeanes *et al.* (82). Isomaltotriose has also been obtained by Wolfrom & Thompson on partial hydrolysis of beef liver glycogen (70). By the transfer of glucosyl residues from sucrose to methyl  $\alpha$ -D-glucopyranoside, catalyzed by the action of the enzyme dextranucrase, Jones *et al.* have prepared the crystalline methyl  $\alpha$ -glucosides of the isomaltose series of saccharides through the pentasaccharide (83). By methanolysis of dextran, Scott & Senti had previously obtained the members of this series as anomeric mixtures of their methyl glycosides (84).

In the  $\beta$ -1 $\rightarrow$ 6-linked series Schaffer & Isbell have prepared the isotopically labelled disaccharide, gentiobiose-1- $C^{14}$  (85).

Most of the theoretically possible glucose disaccharides containing the pyranose linkage have been reported, but there continues to be an interesting absence of reports on glucose disaccharides or oligosaccharides containing the glucofuranosyl linkage.

*Other homosaccharides.*—The  $\beta$ -1 $\rightarrow$ 4-linked xylose saccharides have been prepared in crystalline state through the heptaose by Whistler & Tu (86), and the  $\beta$ -1 $\rightarrow$ 4-linked mannose di- and trisaccharides have been isolated by Jones & Painter following the partial hydrolysis of loblolly pine wood (87).

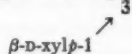
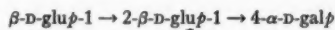
Gorin & Perlin isolated an  $\alpha$ -1 $\rightarrow$ 2-linked mannopyranose trisaccharide after acetolysis of *Saccharomyces rouxii* (88). Two groups of workers have isolated the  $\beta$ -1 $\rightarrow$ 4-linked D-glucosamine di- through pentasaccharides. These substances were obtained by the partial hydrolysis of chitin or deacetylated chitin (89, 90).

A number of arabinose disaccharides are now known, two of the non-reducing type. Rice prepared one of these, apparently  $\alpha$ -D-arabinopyranosyl  $\alpha$ -D-arabinopyranoside, by allowing D-arabinose to stand in 20 N sulfuric acid (91). Partial hydrolysis of natural gums is the most common source of L-arabinose disaccharides. Peach and cherry gums yielded  $\beta$ -L-arabinopyranosyl-(1 $\rightarrow$ 3)-L-arabinose (92); *Virgilia gum*,  $\alpha$ -L-arabino-pyranosyl-(1 $\rightarrow$ 5)-L-arabinose (93). Sugar beet araban has yielded the 1 $\rightarrow$ 3 and 1 $\rightarrow$ 5 L-arabinose disaccharides having the furanosyl type of linkage between the monosaccharide units (94). The anomeric designation of these linkages is, at present, uncertain.  $\beta$ -L-Arabinopyranosyl-(1 $\rightarrow$ 4)-L-arabinose is known as an acid reversion product of L-arabinose (93).

*Heterologous saccharides.*—The partial hydrolysis of corn hull hemicellulose has yielded  $\alpha$ -D-xylopyranosyl-(1 $\rightarrow$ 3)-L-arabinose and  $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-xylose (95).  $\beta$ -D-Galactopyranosyl-(1 $\rightarrow$ 5)-L-arabinose, isolated from this source (96), has been synthesized (97). Peach and cherry gums have yielded  $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4 or 5)-L-arabinose (92).  $\beta$ -D-Glucopyranosyl-(1 $\rightarrow$ 4)-D-mannose and the correspondingly linked manno-pyranosyl glucose have been obtained by the partial hydrolysis of loblolly pine wood (87). An interesting substance containing the disaccharide lactose in combination with O-acetylactaminic acid has been found by Kuhn & Brossmer in milk and colostrum. These workers also have found that lactaminic acid (O-sialic acid,  $C_{11}H_{19}O_8N$ ) cleaves with heat to produce N-acetylglucosamine (98). Other complex saccharides isolated from human milk by Kuhn and co-workers were mentioned in these pages last year (99).

Enzymatic syntheses of a reducing isomer of sucrose,  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 5)-D-fructose (leucrose) (100), and a sucrose terminated trisaccharide,  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -D-glucopyranosyl  $\beta$ -D-fructofuranoside (101), have been described.

The structure of the tetrasaccharide moiety of  $\alpha$ -tomatine, lycotetraose, XVI, has been determined by Kuhn, Low & Trischmann (102). These workers have also reported, with assigned trivial names, several of the di- and trisaccharides constituting lycotetraose. One of these, called lycobiose, is



XVI

$\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-galactose. Since this disaccharide has the reverse of the lactose structure one can be grateful that the name tcalose was

not proposed; however, it appears worth while to recall a previous caution against the assignment of trivial names to the multitude of oligosaccharides currently being isolated (59). The isomeric  $\alpha$ -linked disaccharide  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-galactose, has been isolated from the type VIII pneumococcus-specific polysaccharide (103). The disaccharide primverose,  $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 6)-D-glucose has been isolated from a new source, along with a fructosylglucose,  $[\alpha]_D^{20} = 21^\circ$  (104). Acetolysis of the mucopolysaccharide from hog mucin gave, after partial deacetylation and fractionation, a trisaccharide, D-galactosaminy-(1 $\rightarrow$ 4)-D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucosamine di-N-acetate, in addition to the disaccharide, N-acetylactosamine, previously identified (99, 105). French has recently reviewed the raffinose family of oligosaccharides which are not considered in this section (106).

*Uronic acid-containing oligosaccharides.*—D-Galactopyranosyluronic acid-(1 $\rightarrow$ 2)-L-rhamnose, 4-O-methyl-D-glucopyranosyluronic acid-(1 $\rightarrow$ 4)-D-galactose, and a trisaccharide, D-galactopyranosyluronic acid-(1 $\rightarrow$ 2)-L-rhamnopyranosyl-(1 $\rightarrow$ 4)-D-galactose have been isolated from *Khaya grandifolia* gum (107). Derivatives of  $\alpha$ -D-glucopyranosyluronic acid-(1 $\rightarrow$ 2)-D-xylose have been prepared which identify this substance as the acid obtained by partial hydrolysis of corn hull hemicellulose (108) and chagual gum (109). A similar (or identical) acid has been obtained from peanut hemicellulose (110). The monomethyl ether, 4-O-methyl- $\alpha$ -D-glucopyranosyluronic acid-(1 $\rightarrow$ 2)-D-xylose, has been obtained from hemlock hemicellulose (111). Cellobiuronic acid,  $\beta$ -D-glucopyranosyluronic acid-(1 $\rightarrow$ 4)-D-glucose has been obtained by Jones & Perry from the type VIII pneumococcus specific polysaccharide (103). The serological cross reactions shown by the types III and VIII polysaccharides may be due, in part, to the fact that both contain this disaccharide unit (112). Green has recently reviewed earlier work on the aldbiuronic acids, the uronic acid-containing disaccharides (113).

#### GLUCOSE POLYSACCHARIDES

Since the glucose polysaccharides include cellulose, starch, glycogen, and bacterial dextrans, each with a voluminous literature, an attempt will be made to limit this section to items of general interest.

*Starch and glycogen.*—The evidence presented by Wolfrom & Thompson (70, 115) and others (69) for the existence of small amounts of  $\alpha$ -1 $\rightarrow$ 3-linkages in starch and glycogen will necessitate some revision of the concept of the structure of these substances, built up during the past decade. The isolation of small fragments containing the new linkage is a qualitative method not well adapted to the estimation of how much of the linkage was present in the original material. Nor is the classical methylation procedure most effective in the solution of such a problem. Abdel-Akher *et al.* had reported in glycogen about 1 per cent, in amylopectin, 0.5 per cent, of glucose units resistant to periodate, and they had suggested that these groups might be linked through the 3-position (116). If this proves to be

correct then the number of 1→3-linkages in amylopectin and glycogen would amount to approximately 1/10th of the total chain branching. The possible effect that a substantial number of 1→3 linkages might have upon the interpretation of data relating to the fine structure of glycogen as determined by enzymatic degradation methods (117) will have to be considered.

Liddle & Manners have assayed 25 different glycogen preparations from a variety of sources using  $\beta$ -amylase and potassium periodate to arrive at estimates of the average exterior and interior chain lengths (118). In most instances the exterior chain lengths fell in the range of 7 to 10 glucose units, the interior, 3 to 6. In connection with the length of the interior chains, the isolation of isomaltotriose after the partial hydrolysis of glycogen is most readily interpreted as an indication that chain branching may sometimes occur on adjacent glucose units (70). A new end-group assay has been suggested for amylopectin which should be equally applicable to glycogen and the limit dextrins (119). In this method periodate oxidation is followed by a chemical reduction, the modified polymer is hydrolyzed, and the glycerol separated and determined, each mole of glycerol corresponding to a mole of nonreducing end-group.

There appear to be divergent effects of the extraction procedure upon the molecular size of glycogen. Stetten and co-workers, using a trichloroacetic acid purification, obtained glycogen samples ranging in molecular weight from  $11$  to  $80 \times 10^6$ ; while the conventional potassium hydroxide purification gave weights ranging from  $1$  to  $6 \times 10^6$  (120). Greenwood & Manners obtained glycogen in the latter weight range whether extracted by hot water or by hot potassium hydroxide (121). To this reviewer it seems likely that hot, strong potassium hydroxide could have a degrading action on native glycogen and that Lazarow's "particulate glycogen" observations (122) remain a challenge to workers interested in native glycogens.

Montgomery (123), and Cifonelli, Montgomery & Smith (124) have investigated the precipitation reaction between glycogen and concanavalin A, and have proposed some useful assay methods for glycogen. Larner *et al.* have investigated the enzymatic degradation of various sized glycogen molecules, finding that the attack occurs first upon the molecules of highest molecular weight (125).

The comparison of the oxidation of methyl 4-O-methyl- $\alpha$ -D-glucopyranose and amylose by alkaline hypochlorite represents an interesting approach to the problem of the chemical behavior of complex polysaccharides by studying the comparative behavior of a correspondingly substituted monosaccharide unit (126). Wolff *et al.* showed that the formylation of starch is a reversible process which reaches a maximum degree of substitution at approximately 2.3 formyl groups per glucose unit (127).

*Other glucose polysaccharides.*—The *in vitro* synthesis of cellulose by a cell-free enzyme system from *Acetobacter xylinum* has been reported by Greathouse (128). The optimum activity of the system was found to occur at pH 8.5 to 9. However, appreciable synthesis occurred over the range from

6.5 to 9.5. Adenosine triphosphate was required in rather large amounts by the system. When glucose-1-C<sup>14</sup> was added 96 per cent of the radioactivity of the polymer was found to be retained at position one. This author also explored the effect of adding glycerol-1,3-C<sup>14</sup> and glucose-6-C<sup>14</sup> during the incubation period. With the latter substance 82 per cent of the label was retained in position six, whereas with the former, the label was distributed among all six positions with minor amounts being found at positions 2 and 5. Since these reports were not of a preliminary nature it is remarkable that they fail to include some evidence characterizing the synthesized polymer as cellulose. Aside from the statement that it yields glucose on hydrolysis, no properties of the polymer were reported. When substantiated by adequate characterization of the synthesized substance as cellulose this work will constitute an important milestone in cellulose chemistry.

Falconer & Purves observed a high degree of selectivity in the removal of the nitrate group at position 2 in the denitration of cellulose nitrate (129). Using hydroxylamine in pyridine they obtained a nitrate having 1.7 substituents per glucose unit in which 94 per cent of the hydroxyl groups at position 2 were free. This was proved by methylation followed by complete denitration and hydrolysis to 2-O-methylglucose in good yield. A similar result was obtained by Honeyman & Stening in the preferential removal of the 2-nitrate from methyl 4,6-O-ethylidene- $\alpha$ -D-mannoside 2,3-dinitrate by sodium methoxide (130). It may be a property of nitrate groups adjacent to the glycosidic carbon atom to be readily removed, but such pronounced selectivity among reactive positions has seldom been observed in cellulose reactions.

The bacterial dextrans present a great challenge to workers interested in finding relatively simple methods for the assay of the different types of linkages which may occur in glucose polysaccharides. It would be a very laborious undertaking to attack such a large number of substances using the classical methylation techniques. However, this approach has been employed by Van Cleve, Schaefer & Rist on NRRL B-512 dextran, one of the simpler and more important dextrans (131). The results showed 5 per cent 1,3-linked units, the remainder 1,6-linked, in good agreement with the results of periodate assay, and the further information not given by the latter method, that the 1,3-linkages occur at branch points. The periodate assay, as developed by Jeanes and co-workers (132), groups terminal and 1,6-linked units by their consumption of two moles of reagent and their yield of one mole of formic acid. The 1,3-linked units are characterized by their failure to react with the reagent. The 1,2- and 1,4-linked units are groups together, as "1,4-like," by their reaction with one mole of reagent and failure to produce formic acid. Scott, Hellman & Senti have employed optical rotation in cuprammonium solutions to resolve the "1,4-like" units into the proportions of 1,2-, and 1,4-linked units (133). They have also found indications the NRRL dextrans B-1149, and B-1355 fraction S, contain some linkages other than 1,6 in linear portions of the polysaccharide chain.

Using a combination of techniques, including periodate oxidation and methylation, Chanda, Hirst & Manners assayed the linkages in lichenin and isolichenin from Iceland moss (134). They found lichenin to be a linear polymer containing 30 per cent  $\beta$ -1,3-, and 70 per cent  $\beta$ -1,4-linkages. Isolichenin was found to be a relatively short-chain linear polymer containing 60 per cent  $\alpha$ -1,3-, and 40 per cent  $\alpha$ -1,4-linkages.

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## NEWER DEVELOPMENTS IN RELATION TO PROTEIN BIOSYNTHESIS<sup>1,2</sup>

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### THE GENETIC CONTROL OF PROTEIN SYNTHESIS

From studies on inborn errors of metabolism [Garrod (1)], on the heredity of the anthocyanin pigments of flowers [Lawrence & Price (2)], on the development of eye pigments in *Drosophila* [Ephrussi (3)], and on auxotroph mutants in microorganisms [Beadle (4); Horowitz (5)], the idea emerged that the synthesis of each enzyme or specific protein is controlled by one gene. The success of the one gene-one enzyme theory in unravelling many pathways of metabolism in microorganisms, and its confirmation by transduction and transformation experiments, made the theory so familiar that it is often considered by biochemists not only as an axiom but as accounting for all the facts of genetics. This is grossly exaggerated, but one may safely admit that the one gene-one protein hypothesis describes a fundamental aspect of the chemical basis of heredity in man as well as in microorganisms. The researches of Pauling *et al.* (6, 7) on sickle-cell anemia and of Kalckar, Anderson & Isselbacher (8) on galactosemia are two recently studied examples of the basic relation between the presence of a protein and that of the corresponding gene in man.

When the gene mutates the normal protein is not formed but, in certain cases at least, an abnormal and closely related protein is found in the mutant. Enzymes differing in their thermostability have been found in alleles of *Escherichia coli* [Maas & Davis (9)] and of *Neurospora* [Horowitz & Fling (10)]; a mutation resulting in a change of the optimal temperature of activity of a dehydrogenase has also been reported [Fincham (11)]. Suskind, Yanofsky & Bonner (12) compared several strains of *Neurospora* corresponding to different mutations within the locus of tryptophan synthetase. All the mutants are deprived of the enzyme but several of them each contain a protein which cross-reacts with the antiserum prepared against tryptophan synthetase, and which behaves like the normal enzyme in the course of purification. Mutation of a gene may thus effect a slight change in the protein produced and not necessarily the complete inhibition of the synthesis of a type of protein. The appearance of sickle-cell hemoglobin instead of

<sup>1</sup> The survey of the literature pertaining to this review was completed in August, 1957.

<sup>2</sup> The following abbreviations are used in this chapter: AMP for adenosine monophosphate (adenylic acid); ATP for adenosine triphosphate; CTP for cytidine triphosphate; DNA for deoxyribonucleic acid; GTP for guanosine triphosphate; RNA for ribonucleic acid; and UTP for uridine triphosphate.

normal hemoglobin results from a single mutation. Ingram (13) has shown that hemoglobin from patients with homozygous sickle-cell anemia differs from normal hemoglobin by the replacement of one glutamic acid by one valine residue in the polypeptide chain, and this seems to be the only difference in a polypeptide made of some 300 amino acid molecules. This very important result establishes that a typical Mendelian mutation can cause the replacement of one amino acid by another one in a specific protein, and it gives for the first time an experimental support to the hypothesis that the genetic material controls the assortment and the arrangement of amino acids in polypeptide chains.

One can consider as well established that DNA is the genetic material responsible for the synthesis of the individual proteins [see, e.g., the review by Hotchkiss (14)]. It is thus reasonable to infer that the structure of a protein must bear some simple relationship to that of the DNA of the corresponding gene. Since DNA and proteins are linear polymers, the simplest form of this idea is that a given arrangement of the nucleotides in DNA strictly determines a unique arrangement of the amino acids in the corresponding protein. It is generally assumed implicitly that the nuclear gene carries all the information required for organizing a protein molecule, but in fact it can only be stated that the nuclear gene provides the information that the rest of the cell lacks for making the corresponding protein, and cases are known where the synthesis of specific proteins depends on both Mendelian and cytoplasmic hereditary factors [Ephrussi (15)]. One may thus wonder whether the nuclear genes contain a complete blueprint for each protein or whether they control only the synthesis of part of each polypeptide chain.

The possibility of detecting in microorganisms and phages genetic recombinations which occur with an extremely low frequency has permitted a very fine analysis of the structure of the gene down to molecular level. Benzer (16, 17), Pontecorvo & Roper (18, 19, 20), and Jacob & Wollman (21) have been able to estimate that the size of a section of the genetic material corresponding to the synthesis of a protein is that of a few thousand nucleotide pairs. If one assumes with Crick, Griffith & Orgel (22) on numerical grounds that each amino acid in a protein chain should require three nucleotides in DNA to be determined unequivocally, a gene would be large enough to control the arrangement of around one thousand amino acids, which is the order of magnitude of a large polypeptide chain. If the nucleotide triplets are partially overlapping [Gamow, Rich & Yčas (200); Brenner (202)], the number of amino acids arranged may even be greater. It does not seem impossible, therefore, according to these estimations, that the arrangement of all the amino acids of a protein is controlled by the arrangement of the nucleotides in the DNA of the corresponding gene. The crucial test will possibly require a comparison of the structure of a protein with that of the corresponding DNA. This experiment will probably be

performed in the near future since the tools are at hand, namely, chemical methods for determining the sequence of the amino acids in polypeptides and genetic analysis accurate enough to locate the position of a mutation inside a gene within distances of the order of a few nucleotide pairs. It may also be hoped that chemical methods for establishing the sequence of nucleotides in DNA will be developed soon [Jones & Letham (207); Shapiro & Chargaff (208)]. A very fertile field of investigation is now opened for biochemists interested in amino acid sequence, and important discoveries will certainly result from their collaboration with biologists. The striking resemblances between several proteins secreted by the same organ [Harris & Lerner (23)] also offer an opportunity for finding out whether each of these proteins is synthesized independently from end to end as expected from template theories, or whether they share some common oligopeptide precursors and are made stepwise according to the views of Syngé (24), Fruton (25), and Anfinsen and colleagues (26, 27, 28).

Since the nuclear genetic material ultimately controls the synthesis of the individual proteins, one may wonder whether the nuclear genes participate directly in the making of proteins or whether they control indirectly the protein synthesizing machinery. The most direct approach to this problem has been the study of the effects of enucleation on the synthesis of cytoplasmic proteins. Two unicellular organisms have been used for studies of this type: a green marine alga, *Acetabularia mediterranea*, and a protozoan, *Amoeba proteus*. These organisms when cut into nucleate and nonnucleate halves can survive for a considerable time. Hämmerling (29) observed in 1934 that nonnucleate *Acetabularia* fragments are capable of growth and morphogenesis. Biochemical studies by Brachet and collaborators have now shown clearly that protein synthesis continues undisturbed in the cytoplasm for a long time after enucleation of the algae. Thus the rate of incorporation of  $\text{CO}_2$  or of glycine into proteins is the same in enucleated as in nucleate parts up to two weeks after cutting the alga [Brachet & Chantrenne (30); Brachet, Chantrenne & Vanderhaeghe (31)]. Net synthesis of cytoplasmic proteins is also independent of the presence of the nucleus [Vanderhaeghe (32)]. Recently, Baltus (33) established that the formation of one specific protein, aldolase, goes on at normal speed in nonnucleate parts of *Acetabularia*. Nevertheless, protein synthesis in *Acetabularia* cannot be regarded as completely independent of the nucleus, for a change occurs in the nonnucleate fragments about two weeks after the algae have been cut. Between the twelfth and the fifteenth day after section, net synthesis of protein stops, although the fragments survive for two months thereafter [Brachet & Chantrenne (30); Brachet, Chantrenne & Vanderhaeghe (31); Vanderhaeghe (32)]. It would appear that some substance which is required for protein synthesis and which is produced by the nucleus had been exhausted in the cytoplasmic fragments. It is conceivable that this substance is a nonspecific product of the nuclear metabolism, but it might also be a specific

product of the genetic apparatus [for discussion of this point, see Brachet (34, 35, 36); Brachet & Chantrenne (37); Chantrenne (194)]. With *A. proteus* it is not possible to study net protein synthesis in enucleate fragments, for these very rapidly lose the ability to move about and to catch prey. They are actually starving and they survive for 12 days or so by consuming their own substance, including some of their proteins. Nevertheless, Mazia & Prescott (38) and Brachet & Ficq (39) were able to measure the incorporation of labelled amino acids into their proteins. Although enucleation caused an early drop in the rate of labelling of the proteins, protein synthesis continued in the absence of the nucleus for some time.

Many changes result from enucleation in *Amoeba*. For instance, the uptake of free amino acids is reduced, although less than protein synthesis [Mazia (191); Mazia & Prescott (38)], along with the loss of glycolysis and of the ability to use the stores of polysaccharides and lipides [Brachet (40, 41, 42)]. It is little wonder then that protein synthesis also suffers, and the early drop in amino acid incorporation observed after enucleation does not necessarily mean that the nucleus is directly involved in the production of some cytoplasmic proteins. Enucleation experiments on newt eggs have been briefly reported by Tiedemann & Tiedemann (43); here again no difference in the rate of  $\text{CO}_2$  incorporation into the proteins was observed between nucleate and enucleate cytoplasm. Studies on mammalian reticulocytes, which are naturally occurring enucleate cells, also show that cytoplasm is able to incorporate amino acids into its proteins [London, Shemin & Rittenberg (44); Borsook *et al.* (45)] and even to synthesize hemoglobin [Nizet & Lambert (46); Koritz & Chantrenne (47); Kruh & Borsook (48)]. It is clear that the cytoplasm can keep for various periods of time all that is necessary for protein synthesis, including the genetic information. The control exerted on the synthesis of cytoplasmic proteins by the nucleus is indirect and remote, and DNA is not involved *per se* in the synthesis of cytoplasmic proteins. On the basis of these results, one can exclude any idea of DNA being the template upon which amino acids are assembled to form cytoplasmic proteins.

This of course does not give any information about the function of the genetic material in the formation of nuclear proteins. Mirsky, Allfrey & Osawa (49 to 53) made an extensive study of amino acid incorporation into the proteins of isolated thymus nuclei. They observed that protein synthesis virtually ceases when DNA is removed from the nucleus and that amino acid uptake resumes when DNA is restored. Protein synthesis in nuclei is inhibited by deoxyribonuclease and is insensitive to ribonuclease or chloramphenicol, which are strong inhibitors of protein synthesis in many systems. Logan & Ficq (54), repeating the experiments of Mirsky's group with an autoradiography technique, confirmed the fact that the incorporation occurs in the nucleus and not in any contaminating cytoplasm or intact cells; they have also confirmed the inhibition by deoxyribonuclease, the

restoration by DNA, and the fact that ribonuclease exerts but a small inhibition. Logan (55) was able to isolate rat liver nuclei which incorporate amino acids into their proteins *in vitro* and behave in the same way as thymus nuclei in many respects.

All these experiments establish that protein synthesis takes place in the nucleus as well as in the cytoplasm. The effects of deoxyribonuclease and DNA at first seemed to show that DNA is directly involved in the synthesis of some nuclear proteins. However, the effect of DNA is not specific, for Mirsky, Osawa & Allfrey (52, 53) showed that DNA from other tissues, denatured or partially degraded DNA, and even RNA, are able to replace DNA in restoring the amino acid incorporation. These puzzling observations have now been clarified by Allfrey & Mirsky (56), who have shown that the removal or destruction of DNA suppresses ATP synthesis in isolated thymus nuclei and that ATP production by oxidative phosphorylation is restored by the same substances which restore amino acid incorporation, namely DNA, split products of DNA, RNA, and polyadenylic acid. The observed effects of DNA are thus explained by its being involved—in some unknown way—in ATP formation in isolated nuclei, and there is no evidence left that DNA is directly involved in protein synthesis, even in the nucleus itself, at least in higher organisms.

Gale & Folkes (57 to 61) showed that the removal of nucleic acids from disrupted *Staphylococcus aureus* suppresses amino acid incorporation into proteins and that the addition of intact staphylococcal DNA restores the activity whereas DNA from other sources is ineffective. This fact would seem to prove that the presence of specific DNA is required for protein synthesis. A very disturbing fact, however, is that the reactivation of the system can also be obtained with substances, as yet unidentified, found among the ribonuclease-split products of yeast RNA. The meaning of these results is not clear at present [Gale (59, 61)], and further experimental data are needed before final conclusions can be drawn.

More recently Spiegelman (62) was able to remove all the DNA from osmotically shocked protoplasts of *Bacillus megaterium* without inhibiting enzyme synthesis. However, an extensive and very rapid resynthesis of DNA occurred, which prevents the formulation of definite conclusions on the importance of DNA in this system.

In itself, the synthesis of DNA is not necessary for protein synthesis in bacteria. Thymidine-requiring strains can make proteins [Jeener & Jeener (63)] and even induced enzymes [Cohen & Barner (64)] when DNA synthesis is prevented by the lack of thymidine. Bacteria and yeast which have received high doses of x-rays or substances which inhibit DNA synthesis are capable of enzyme synthesis [Baron, Spiegelman & Quastler (65); Chantrenne & Devreux (124)].

To sum up, experiments on enucleate cytoplasm clearly show that the synthesis of proteins occurs in the absence of the nuclear genetic material,



and the study of isolated animal nuclei and of bacterial systems has not provided evidence for the direct participation of DNA in protein synthesis. A possible exception to this last statement is, however, provided by an interesting study on the effect of  $P^{32}$  decay on enzyme synthesis in *E. coli* [McFall, Pardee & Stent (66)]. Bacteria labelled in their DNA with  $P^{32}$  of very high specific radioactivity lose both the capacity to reproduce and to synthesize enzymes (constitutive as well as adaptive) as their DNA is damaged by  $P^{32}$  decay. The inactivation occurs as soon as a few hundred  $P^{32}$  atoms contained in the DNA of a bacterium have decayed. The conclusion drawn from these experiments is that the entire bacterial nucleus acts as a unit in controlling the formation of specific proteins and that the integrity of DNA is required for enzyme synthesis in *E. coli*. The discrepancy between the conclusions of these experiments and the data from enucleation experiments in higher organisms is not easily explained. Chromosomal organization seems to be more involved in higher organisms than in bacteria and there are reasons to believe that the delegation of metabolic functions from the nucleus to cytoplasmic structures is not as advanced in bacteria as in more complex organisms [Pontecorvo (18)].

It is clear that, in higher organisms at least, DNA controls protein synthesis indirectly and one wonders how the genetic action is transmitted from the genes to the protein-synthesizing systems of the cytoplasm. As the general trend at present is to consider that the genetic information resides in the arrangement of the nucleotides in DNA, the question becomes: To what substance is this information transferred? No doubt the more likely candidate for such a function is RNA, which has been known for a long time to play a part in protein synthesis, but whose function is not yet clear [see Brachet (67)]. RNA would probably be able to carry the genetic information since the RNA extracted from certain viruses is able to transmit infection. The general question of RNA specificity is raised by its ability to function in this manner.

#### RNA AND PROTEIN SYNTHESIS

*Are specific ribonucleic acids involved in protein synthesis?*—Ribonucleic acids from different origins differ in composition and in physical properties, and it would be as misleading to consider RNA as one unique chemical substance as to believe that all proteins are identical. Unfortunately the resolving power of the present methods for separating different RNA's is very poor and there is no way of testing RNA preparations for homogeneity. Nor is there any biological test for distinguishing between several ribonucleic acids, except possibly immunological procedures [Masamune *et al.* (68)].

A notable exception of course is the RNA of a few viruses. It has been convincingly shown (see review by G. Schramm in this volume) that RNA extracted from a virus is able to cause the production of specific proteins (virus proteins) when introduced into competent cells. This establishes that

specific RNA's do exist which are able to influence protein synthesis in a specific way. This situation may not be restricted to the case of viruses, for results obtained in several laboratories suggest that RNA extracted from bacteria might induce the production of enzymes in homologous cells in the absence of the usual inducers. Thus Minagawa (69, 70) was able to confer copper resistance to yeast cells by adding RNA extracted from copper-adapted cells. Hunter & Butler (71) induced  $\beta$ -galactosidase formation in *B. megaterium* with RNA preparations extracted from a lactose-adapted culture of the same organism. Reiner & Goodman (72) had reported before somewhat similar results for gluconokinase formation in *E. coli*. Kramer & Straub (73) also reported that RNA extracted from a *Bacillus cereus* strain which produces penicillinase as a constitutive enzyme can induce a limited production of this enzyme in an adaptive strain.

These are all very interesting results. The trouble is that in most cases they are not easily reproducible. A careful critical analysis of the experimental conditions will be necessary before their meaning can be appreciated. If they prove to be correct, the experiments quoted above will acquire an importance comparable to that of bacterial transformation by DNA.

*Is the integrity of RNA required for protein synthesis?*—Ribonuclease has been shown to enter many types of living cells and to disturb cell functions in various ways. Thus Lansing & Rosenthal (74) observed that ribonuclease changes the permeability of plant cells to ions. Kaufmann *et al.* (75, 76) reported mitotic abnormalities in roots dipped in a ribonuclease solution. The enzyme also inhibits cell division in amphibian eggs [Brachet & Ledoux (77)], in various tumors [Ledoux & Baltus (78); Ledoux (79); Hadjiolov & Zacharieva (80)] and in tissue cultures [Firket, Chèvremont & Chèvremont (190)].

Using ribonuclease, one may thus hope to degrade RNA within the living cell and to test the effect of RNA destruction on protein synthesis. Ribonuclease was indeed found to inhibit protein synthesis almost completely in onion roots and in *Amoeba* [Brachet (81 to 84)], in bacteria [Groth (85); Jeener (86); Nisman *et al.* (87); Kramer & Straub (73)], and in bacterial protoplasts [Lester (88); Beljanski (89); Spiegelman (62); Bridoux & Hanotier (90); Fraser & Mahler (91)]. In onion roots and in *Amoeba* at least, the enzyme does not inhibit respiration or phosphorylation or the uptake of amino acids by the cells, but it does prevent amino acid incorporation into protein molecules [Brachet (82, 83, 84)]. Its action must therefore bear rather directly on protein synthesis. Onion roots do not lose more than 20 per cent of their RNA under the action of ribonuclease; this indicates that a limited damage to RNA, or the destruction of certain RNA fractions only, is enough to suppress protein synthesis.

It is not certain, however, whether ribonuclease always exerts its inhibitory effect on protein synthesis by splitting RNA. In onion roots [Brachet (81)] and in ascites cells [Ledoux (78)] ribonuclease first stimulates RNA



synthesis, and there are indications that the RNA thus synthesized in ascites cells contains an excess of pyrimidines [Ledoux & Vanderhaeghe (92)]. On the other hand, Brachet (93) observed that various basic proteins can inhibit amino acid incorporation into proteins of onion roots, although to a lesser extent than ribonuclease does. Quite similar results have been reported by Hultin (94) studying liver homogenates. These observations suggest that basic proteins, including ribonuclease, might form complexes with RNA and interfere in this way with its function in protein synthesis; this might explain part of the action of ribonuclease, which is, however, much more inhibitory than other basic proteins. Ribonuclease has also been shown to cause the liberation of hydrolytic enzymes in homogenates of sea urchin eggs [Lundblad & Hultin (95)] and *Tetrahymena* [Roth (96)]. This, however, does not occur in living *Amoeba* [Brachet (97)]. Under certain conditions ribonuclease may also cause secondary effects, e.g., the destruction of the protoplasts [Brenner (98)]. All this shows that the effect of ribonuclease on living cells is often difficult to interpret, but it is clear that with several types of cells the first mechanisms to be disturbed seem to be RNA and protein synthesis. Unexplained differences of action have been observed between apparently pure ribonuclease preparations of different origins [Ledoux & Brachet (99)].

Under carefully controlled conditions Landman & Spiegelman (100) were able to remove a large part of the RNA from protoplasts of *B. megaterium* without destroying these structures; they observed that destruction of RNA resulted in a complete inhibition of enzyme synthesis. Gale (59, 60, 101) and Gale & Folkes (57, 58) removed RNA from disrupted *S. aureus* by various treatments. The properties of their preparations depended very much on the degree of nucleic acid removal: certain preparations which lost, together with nucleic acids, the capacity of synthesizing enzymes could be reactivated by RNA, some of them only with RNA from the same bacteria. But mixtures of purines and pyrimidines or RNA hydrolysates also restored enzyme formation, and this may indicate that protein synthesis requires a RNA which can be resynthesized under appropriate conditions in these preparations.

Using "shockates," that is, protoplasts from *B. megaterium* submitted to a controlled osmotic shock, Spiegelman (62) succeeded in removing selectively either RNA or DNA. The effects of these treatments upon enzyme synthesis led him to conclude that the physical integrity of RNA molecules is all important for enzyme formation.

A similar conclusion has been drawn from experiments on the effects of analogues of the natural purines and pyrimidines. When the RNA of plant viruses has incorporated thiouracil or azaguanine the infectivity of the virus is very much reduced [Jeener & Rosseels (102); Jeener (103); Matthews (104, 105); Mandel, Markham & Matthews (106)] and it is clear that the abnormal RNA is unable to fulfill its function. Similarly, azaguanine is

incorporated into the RNA of various bacteria [Lasnitski, Matthews & Smith (107); Matthews & Smith (108); Mandel, Sugarman & Apter (109)] and it has been shown that in *B. cereus* azaguanine can replace as much as 23 per cent of the RNA guanine [Matthews & Smith (108)]. Azaguanine also causes a partial inhibition of growth but the synthesis of certain enzymes is completely inhibited [Creaser (111, 112)]. It is not known, however, whether this inhibition results from the formation of abnormal RNA or from an effect of azaguanine on some smaller nucleotidic compounds [Mandel (110)].

Taken together, the data reviewed above strongly suggest that the integrity of RNA or at least of certain RNA fractions is a necessary condition for protein synthesis.

*Is the synthesis of RNA required for enzyme formation?*—As early as 1952, Gale & Folkes (113) had observed that a supplement of purines and pyrimidines stimulates protein synthesis in *S. aureus*. Creaser (114) later made similar observations for the induced synthesis of  $\beta$ -galactosidase in the same organism. Using pyrimidineless mutants of *E. coli*, Pardee (115) observed that adaptive enzyme formation is possible only if exogenous pyrimidines are supplied, except under special conditions when destruction of RNA probably makes these bases available to the cell [Pardee (116)]. These experiments, which may be interpreted as showing that RNA synthesis is required for protein synthesis, were not quite convincing, for in *E. coli* there is often a close connection between growth and adaptive enzyme synthesis [Monod, Pappenheimer & Cohen-Bazire (117); cf., however, Mandelstam (118)]. The dependence of new enzyme formation on nucleic acid precursors was also shown by Spiegelman, Halvorson & Ben-Ishai (119) for resting yeast, where the ability to form  $\alpha$ -glucosidase is strongly dependent upon the level of the free nucleotide pool. The best way to deplete the pool is actually to cause yeast to make proteins by supplying amino acids.

Low concentrations of 5-hydroxyuridine inhibit for some time the formation of  $\beta$ -galactosidase in *E. coli* without inhibiting growth. As this analogue is supposed to inhibit RNA synthesis, it has been inferred that the synthesis of new RNA molecules is necessary for the synthesis of the induced enzyme [Spiegelman, Halvorson & Ben-Ishai (119)]. Since growth was not inhibited under those conditions, it would seem that the formation of all the proteins does not depend to the same extent upon RNA synthesis. Creaser (111, 112) similarly showed that 8-azaguanine and 2,6-diaminopurine inhibit the formation of an adaptive enzyme in *S. aureus* but that the analogues exert a less inhibitory effect on the formation of two constitutive enzymes. These data, as well as Spiegelman's results with 5-hydroxyuridine, have been interpreted as suggesting that the formation of induced enzymes depends on a continuous synthesis of RNA, and that the RNA corresponding to induced enzymes is more labile than the RNA corresponding to constitutive

proteins. This distinction has lost much of its weight, however, after the observation by Gros & Spiegelman [quoted by Spiegelman (62)] that the formation of constitutive  $\beta$ -galactosidase is inhibited by 5-hydroxyuridine as well as the synthesis of the corresponding adaptive enzyme. Similar observations were made in the reviewer's laboratory for penicillinase formation in *B. cereus*; the formation of both induced and constitutive penicillinase is completely inhibited by azaguanine under conditions where growth is reduced by 50 per cent only [Chantrenne & Devreux (120)].

A few attempts have been made to follow directly RNA synthesis during enzyme formation. Thus Gale & Folkes (58) briefly reported that the incorporation of 2- $C^{14}$ -uracil into RNA is increased during the early stages of galactose adaptation in disrupted *S. aureus*. The reviewer [Chantrenne (121, 122)] showed that the induced formation of catalase, cytochrome peroxidase, and cytochrome-*c* in resting yeast is accompanied by an increased synthesis of RNA. This must be related to enzyme synthesis, for the increased RNA synthesis is not observed with fully adapted cells and it is suppressed by the same doses of ultraviolet radiation as the induced formation of the enzymes [Bečarevič (123)]. These results are certainly in line with the idea that RNA synthesis is required for enzyme adaptation. There might be some analogy between the metabolic changes in nucleic acid during enzyme induction and the high turnover of a particular RNA during phage infection [Volkin & Astrachan (192)]. However newer results obtained in the reviewer's laboratory suggest that the increased synthesis of RNA observed during enzyme induction in yeast may be a secondary effect resulting from a sudden increase in the acid-soluble pool which occurs when enzyme induction or protein synthesis begin [Chantrenne & Devreux (124); Gobert (125)].

The incorporation of amino acids into proteins of disrupted cells has been shown to depend on various RNA derivatives. The most extensive studies of this type are those by Gale. Broken *Staphylococci* can be deprived of their nucleic acids to various extents; when the resolution is sufficient, as already mentioned, the preparations lose the ability to incorporate amino acids into their proteins. Under certain conditions the incorporation can be restored by partial hydrolysates of RNA. Certain fractions of the hydrolysate have been shown to activate in a rather specific way the uptake of individual amino acids. Although these incorporation factors have been obtained from ribonuclease digests of RNA, they are not nucleotides or nucleosides and their chemical nature remains to be established [Gale (59, 60)].

Webster (126, 189) reported that microsomes of pea seedlings, which lose the ability of incorporating amino acids *in vitro* after ribonuclease treatment, can recover this capacity in the presence of a mixture of ribonucleosides or nucleoside-5'-phosphates, or better of the di- or triphospho derivatives.  $C^{14}$ -labeled ATP is incorporated into RNA in the same preparations, and amino acid analogues which inhibit protein synthesis also inhibit the

incorporation of the adenosine residue of ATP into RNA. This suggests a close relationship between the two processes.

Although many of the experimental facts quoted above are compatible with the idea that new ribonucleic compounds must be formed during the synthesis of certain enzymes, there is no evidence that the synthesis of a specific RNA is involved. It seems almost certain that some metabolism of RNA precursors plays a part in enzyme formation, but the possibility that it may concern molecules smaller than RNA should not be overlooked.

*Amino acids and RNA synthesis.*—Sands & Roberts (127), Gale & Folkes (113), Borek, Ryan & Rockenbach (128) have observed that, in *S. aureus* and in *E. coli*, amino acid deficiency suppresses not only protein synthesis but RNA synthesis as well. Schmidt *et al.* (129) have reported that in yeast a sulfur starvation or the addition of ethionine prevents the synthesis of both protein and RNA although the formation of purines and soluble nucleotides is not inhibited. From nutritional experiments, Clark, Naismith & Munro (130) concluded that the RNA level in liver depends in some way on amino acid supply.

The relation between amino acids and RNA synthesis in these various cases suggests that RNA synthesis may depend on protein synthesis. However, in *S. aureus* [Gale (101, 113, 131)] and in *E. coli* [Wisselman, Smadel, Hahn & Hopps (132)] chloramphenicol inhibits protein synthesis without preventing RNA formation, and amino acids stimulate RNA synthesis even when protein synthesis is suppressed by the antibiotic [Gale (113)]. The relationships between amino acids, chloramphenicol and RNA formation have now been studied in more detail by Gros & Gros (133, 134) and by Pardee & Prestidge (135) in amino-acid-requiring mutants of *E. coli*, and by Yčas & Brawerman (136) with other microorganisms. RNA synthesis requires the simultaneous presence of all the amino acids, even when protein synthesis is 98 per cent inhibited by chloramphenicol. Under these conditions the addition of a very small amount of the limiting amino acid will cause the synthesis of a large amount of RNA. It could be estimated that one amino acid molecule makes possible the polycondensation of at least eight nucleotide residues [Gros (134)]. There is a small residual protein synthesis, and it cannot be completely ruled out that the formation of some unstable protein involved in RNA synthesis might explain the catalytic effect of the amino acids. But this is unlikely for several reasons [Gros (134)], and it is much more probable that the individual amino acids by themselves catalyse RNA synthesis. It should not be overlooked that the RNA formed under these conditions is abnormal on several accounts: although its composition is apparently normal [Gros & Gros (133); Pardee & Prestidge (135)] its electrophoretic mobility, and the way it is bound to proteins are unusual [Pardee, Paigen & Prestidge (137)]. Whereas normal RNA in *E. coli* is stable, once formed, the RNA built up in the presence of chloramphenicol breaks down when the antibiotic is removed or when

dinitrophenol is added [Neidhart & Gros (138)]. It is not known how closely the mechanism of the synthesis of this abnormal RNA resembles that of normal RNA, but they have at least one point in common, namely the requirement for amino acids. Neither is it known whether this RNA piles up because polypeptides cannot form in the presence of chloramphenicol, or whether amino acids cannot condense because the nucleic acid synthesized in the presence of the antibiotic is abnormal. Whatever the primary effect of the drug may be, it is striking that it causes at the same time a practically complete inhibition of protein synthesis and some subtle change in the structure of the RNA synthesized. One cannot but feel that a fine mechanism involved in the specificity of RNA synthesis has been touched here. It might be that the same delicate mechanism is also disturbed in a particular *E. coli* mutant which piles up RNA in the absence of methionine by some process which is abnormally sensitive to ultraviolet radiation [Borek, Rockenbach & Ryan (139)].

Most of the observations reported above can be accounted for by assuming that RNA and proteins are formed from a pool of common precursors, e.g., amino acids bound to nucleotidic compounds, which, upon condensing, give either RNA or protein. Interesting discussions of this point will be found in the original papers [Gros (134); Yčas & Brawerman (136); Pardee & Prestidge (135, 137)] as well as in review articles by Simkin & Work (140) and by Spiegelman (62). The relation between amino acids and RNA synthesis might be the key to a precise formulation of a template mechanism for the synthesis of proteins and nucleic acids.

#### PATHWAYS OF PROTEIN SYNTHESIS

*Amino acid activation.*—It has long been recognized [Lipmann (141); Linderstrøm-Lang (142); Borsook & Huffman (143)] on thermodynamic grounds that the formation of polypeptides should require activation of the amino acids. Reactions involving the formation of a high energy anhydride bond between AMP and the carboxyl group of several amino acids were discovered a few years ago [Hoagland (144); De Moss & Novelli (145)] and are now being studied in several laboratories (see pp. 561 to 612).

Extracts which catalyse these reactions have been obtained from various animal tissues (144, 146, 147, 148) and from many microorganisms (145, 149, 150). Their presence in plants has been briefly reported [Webster (151)]. A tryptophan-activating enzyme has been isolated from beef pancreas by Davie, Koningsberger & Lipmann (146), while an enzyme which activates methionine has been purified from yeast by Berg (150). Cole, Coote & Work (147) have separated from hog pancreas a fraction specific for tryptophan and another one which activates threonine and serine. Schweet (152) and Koningsberger, Van de Ven & Overbeek (153) have briefly reported the purification of the enzyme specific for tyrosine. It is thus reasonable to think that there might be one specific activating enzyme for each of the amino acids which undergo this type of reaction.

There are good reasons to believe that the amino-acid-activating enzymes play a part in protein synthesis. In liver homogenates the incorporation of amino acids into microsomal proteins requires the presence of a soluble fraction, the "pH 5 enzyme," which is known to contain the amino-acid-activating enzymes [Zamecnik *et al.* (154, 155); Hoagland, Keller & Zamecnik (156); Littlefield & Keller (157); Hoagland, Zamecnik & Stephenson (158)]. The tryptophan-activating enzyme which does not activate any other natural amino acid does activate various analogues of tryptophan which can be incorporated into protein instead of tryptophan, but it does not activate other analogues which are not incorporated [Davie, Koningsberger & Lipmann (146); Sharon & Lipmann (159)].

There is one puzzling point, however; only about half of the natural amino acids have been found to be activated as aminoacyl adenylates, and it is always the same group of amino acids which have been found to react, whatever the source of the enzyme preparation. One may then wonder whether the remaining amino acids are activated by some other process. Even with a very sensitive tracer method, no carboxyl-activated glycine could be detected in yeast [Boeyé (209)]. UTP, GTP, and CTP have not been found to react with amino acids as ATP does, although the existence of nucleotide compounds of amino acids has been reported. Thus Reith (160) has isolated from ascites cells an aspartic-acid derivative containing uridylic acid, and Hansen & Hageman (161) have obtained from chicken liver a compound tentatively identified as adenosinediphosphoglutamic acid. Aspartic and glutamic acids are among the amino acids which have not been found to react with ATP in the presence of tissue or bacterial extracts. Recently, Koningsberger, Van der Grinten & Overbeek (162) have obtained some indication of the existence of peptide-linked nucleotide compounds in yeast.

It is somewhat disturbing that the activity of a tissue extract towards tryptophan or methionine is much higher than for other common amino acids [De Moss & Novelli (149); Davie, Koningsberger & Lipmann (146)]. This might prove to be a result of the isolation procedure, but it remains one of the points to be clarified.

As the amino acid adenylates are very strongly bound to the enzymes and do not dissociate to any measurable extent, they cannot be considered as carriers of activated groups like the various acylcoenzymes A or ATP. It can even be surmised from the properties of such substances that they would have a very short life in the free state. They are mixed anhydrides of a carboxylic acid with a substituted phosphoric group, and substances of this class are known to react very rapidly with amino acids to form a peptide bond [Chantrenne (163 to 167); Katchalsky & Paecht (168, 169); Avison (170); Van de Ven, Koningsberger & Overbeek (171)]. Free aminoacyl adenylates in neutral medium would rapidly polymerize or produce a large variety of peptides by reacting with any amino acid present. The formation of well-defined proteins would be possible only if these aminoacyl adenylates were prevented from reacting at random [Chantrenne (165)]. Their strong binding



to the enzyme might be responsible for this protection [Askonas, Simkin & Work (172)].

*The next step.*—Experiments with animal tissues *in vivo* and *in vitro* have clearly shown that the proteins of RNA-rich microsomes generally incorporate amino acids more rapidly than the other cytoplasmic proteins [see review by Brachet (67)]. More recent work confirms this view [e.g., Hendler (173); Rabinovitz & Olson (174); Simkin & Work (175); Clouet & Richter (176), and Laird & Barton (177); see, however, Ziegler & Melchior (178); Campbell, Greengard & Jones (179); and Bhargava (180)]. Disruption of the microsomes by deoxycholate or concentrated salts, moreover, showed that it is the ribonucleoprotein fraction which incorporates amino acids the most rapidly [Littlefield *et al.* (181); Littlefield & Keller (157); Simkin & Work (175)], and it seems that polypeptides appear first as part of the microsomal ribonucleoproteins.

If the activation of amino acids as aminoacyl adenylates is really the first step of protein synthesis, and if it is true that these compounds do not dissociate, one then wonders how the activated amino acids are transferred to the site of their condensation, the microsomes. The activation of fatty acids and aromatic acids is quite similar to that of amino acids and it is known that the adenylyl-bound acyl residue is transferred to coenzyme A; but coenzyme A does not seem to be the acceptor of aminoacyl residues [Jencks & Lipmann (182)]. Hultin (183, 184) observed the accumulation of amino acid derivatives in the soluble fraction of a liver homogenate; these compounds are formed at the expense of ATP and they can transfer their amino acid moiety to microsomal proteins in the absence of ATP. Hendler (185) also obtained evidence for the accumulation of intermediates of protein synthesis in hen oviduct minces.

New data of very great interest show that the carriers of activated amino acids are most probably polyribonucleotides. Hoagland, Zamecnik & Stephenson (158) have found that the "pH 5 enzyme" preparation contains an RNA of a low molecular weight which binds radioactive amino acids in the presence of ATP and that the bound amino acids can be transferred to microsomal protein in the absence of the activating enzyme, provided GTP is present. Very similar results have been obtained by Ogata & Nohara (186). Holley (187) has found that an exchange of C<sup>14</sup>-labeled AMP with the corresponding moiety of ATP is catalysed by alanine in the presence of the "pH 5 enzyme"; this suggests a transfer of the aminoacyl residue to some acceptor. An important fact is that this exchange is completely suppressed by ribonuclease, showing that the acceptor or some part of the system is susceptible to the enzyme. It is reasonable to think that the acceptor is an oligo- or polynucleotide.

It would seem at present that amino acids are first activated as aminoacyl adenylates bound to the corresponding enzyme, that they are trans-

ferred to an RNA of low molecular weight and thence incorporated into microsomal ribonucleoprotein by a process which is mediated by GTP. The nature of this further step and the function of GTP as well as the function of the microsomal RNA, are still completely obscure [Keller & Zamecnik (188)].

*What next?*—The main problems remain, namely, the condensation of the activated amino acids in the right sequence and the folding of the polypeptide chain into the finished protein. Working hypotheses are slowly taking shape, but the experimental facts are few.

Spiegelman (62) has brilliantly summarized the reasons why a template mechanism seems to be the best guess at present for explaining the synthesis of specific proteins. Discussions on template mechanisms will be found in publications by Haurowitz (194), Borsook (195, 196), Lipmann (197), Caldwell & Hinshelwood (198), Wilkins (204), Dalglish (199), and Loftfield (193). Several papers have been devoted to speculations on coding systems, that is, on the way sequences of four different nucleotides in DNA or RNA may control the arrangement of twenty amino acids [Gamow, Rich & Yčas (200); Crick, Griffith & Orgel (22); Brenner (202)]. These speculations make interesting reading and they will prove valuable if they eventually lead to hypotheses amenable to experimentation. As an illustration, the principle of template process proposed by Crick (203) may be mentioned because it fits rather well with several experimental data. Each activated amino acid would be transferred to a specific oligonucleotide by a regular enzyme reaction [cf. the low molecular weight RNA of Hoagland *et al.* (158) and the effect of amino acids on RNA synthesis (134 to 137)]. These oligonucleotide "adaptors," each carrying the corresponding amino acid, would find their right place on the RNA template by forming hydrogen bonds with the complementary sequences in RNA [Crick, Griffith & Orgel (22); Brenner (202)]. The amino acids would thus be lined up and ready to condense in the correct sequence. It seems possible to test experimentally certain aspects of this hypothetical mechanism.

It should not be overlooked that template processes are not the only possibility and alternative ideas are held by several biochemists [Syngé (24); Fruton (25); Anfinsen *et al.* (26, 27, 28); Simkin & Work (140)].

General reviews on protein synthesis by Borsook (195) and by Loftfield (193) will be read with great interest, as well as the *Symposium on Nucleic Acids and Their Role in Protein Synthesis* of the Biochemical Society (205). A wealth of up-to-date information pertaining to the subjects of the present review will be found in two recent books: *Biochemical Cytology* [Brachet (35)] and *The Chemical Basis of Heredity* [McElroy & Glass (206)].

Many fundamental questions related to protein synthesis remain unsolved. How much information is provided by the genes, how much by the cytoplasm? How is the genetic information transmitted to the protein-



synthesizing system? Are there templates, and if so how do they operate? What is the mode of action of the inducer in adaptive enzyme formation and of the antigen in antibody production? How is the synthesis of specific proteins controlled during growth and differentiation? The search for answers to these and other questions will be the task for future research.

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## BIOCHEMISTRY OF THE PROTEIN HORMONES

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This review will be limited to a discussion of the remarkable progress achieved during the past six years<sup>1</sup> in the isolation and characterization of the pituitary and pancreatic hormones, namely growth hormone, thyrotropin, corticotropin (ACTH), prolactin, follicle-stimulating hormone (FSH), interstitial cell-stimulating hormone (ICSH), vasopressin, oxytocin, intermedin (MSH), insulin, and glucagon. Pertinent reviews of either a general (e.g., 1, 2) or a specific<sup>2</sup> nature have been published in the interim. Other works, particularly recent volumes of *Annual Review of Physiology*, and *Recent Progress in Hormone Research*, contain reviews that emphasize physiological aspects. The aim of the present authors is to avoid undue duplication but at the same time to provide a reasonably integrated picture of the protein hormone field, stressing the recent advances in isolation, structural analysis, and structure-activity relationships.

In the past decade great strides have been made in the methods of protein and peptide isolation and characterization. The impact of newer techniques involving end group and sequential analysis, preparative zone electrophoresis, column chromatography with finely ground adsorbents and resins, and countercurrent distribution has been strongly felt in the protein hormone field. All eleven hormones have been obtained in highly purified form, a step that clearly precedes definitive chemical and biological characterization. The complete amino acid sequence of six of these hormones has been determined. The newer preparative methods have not only been extensively used for purification of the hormones but also have been exploited as sensitive criteria of purity.

### INSULIN

*Homogeneity and molecular weight of crystalline insulin.*—Insulin has probably been studied more extensively than any other protein; both its availability in crystalline form and its recognition as a small protein have encouraged such studies. Several excellent reviews on various aspects of the hormone have been published in the past five years, among them the recent *Ciba Foundation Colloquia on Endocrinology* (3). The critical countercurrent distribution studies of Harfenist & Craig (4) have provided excellent evidence that heterogeneity exists in samples of beef insulin that have been crystallized as many as five times. The distribution patterns obtained from several different insulin preparations in the system, 2-butanol:1 per cent

<sup>1</sup> The present review covers the period from January, 1952, to July, 1957.

<sup>2</sup> Specific reviews may be found under the appropriate sections.



dichloroacetic acid, indicated the presence of the same major component, A, with varying amounts of component B and other minor components. In the best samples, A represented approximately 90 per cent of the starting material. A and B possessed full biological potency; some of the other materials also were biologically active. Later, Harfenist (5) demonstrated that A and B differed by only one amide group.

An important sequel to this excellent work was the determination of the molecular weight of insulin using partial substitution with dinitrofluorobenzene, separation of the products by countercurrent distribution, and analysis of the mono-substituted derivative for the dinitrophenyl group [Harfenist & Craig (6)]. The data provided evidence for a molecular weight of approximately 6500, reinforcing the earlier sedimentation and diffusion studies of Fredericq & Neurath (7) that had indicated a value of about 6000. A great body of data, obtained by physical and amino acid studies, had supported a molecular weight of 12,000. Knowledge of the complete amino acid sequence of insulin, provided by the classical work of Sanger and his colleagues, subsequently provided additional evidence that the minimum molecular weight of insulin is in the range of 6000. Confirmative data for this value have since been published [Kupke & Linderström-Lang (8); Fredericq (9, 10); Rees & Singer (11); Sluyterman (12)]; however, the smallest units ordinarily observed in aqueous acid solution by conventional physical methods have a molecular weight of 12,000.

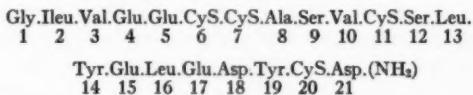
A possible explanation for the confusion over molecular weight is based on the finding by Scott & Fisher (13) and Tanford & Epstein (14) that insulin contains one mole of zinc per 12,000 mol. wt. The unit of mol. wt. 6000 has been observed only under particular conditions that may allow dissociation of the metal. Thus, insulin in solution in the presence of trace amounts of metal ions such as zinc probably exists as a bimolecular association product observed as a unit of mol. wt. 12,000.

*Structural analysis.*—In the period covered by this review, the persistent pioneering efforts of Sanger and his colleagues culminated in the elucidation of the complete amino acid sequence of insulin [Ryle *et al.* (15)]. In 1951 Sanger & Tuppy (16, 17) had reported the amino acid sequence<sup>3</sup> in the phenylalanyl chain of insulin:

Phe.	Val.	Asp.	Glu.	His.	Leu.	CyS.	Gly.	Ser.	His.	Leu.	Val.	Glu.	Ala.	Leu.	Tyr.	Leu.	Val.	CyS.	Gly.
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
										Glu.	Arg.	Gly.	Phe.	Phe.	Tyr.	Thr.	Pro.	Lys.	Ala.
										21	22	23	24	25	26	27	28	29	30

<sup>3</sup> The abbreviations of the amino acid residues are those of Brand, E., and Edsall, J. T., *Ann. Rev. Biochem.*, **16**, 224 (1947). The sequential arrangements are patterned after those suggested by Sanger, F., *Advances in Protein Chem.*, **7**, 1 (1952). Other abbreviations are used as follows: ACTH for corticotropin; FSH for follicle-stimulating hormone; ICSH for interstitial cell-stimulating hormone; and MSH for melanocyte-stimulating hormone (intermedin).

By the application of somewhat similar techniques in the study of the glycyl chain, Sanger & Thompson (18, 19) in 1953 described the structure of the second chain of insulin. Peptides from the partial degradation of the glycyl chain by concentrated acid were separated into simpler mixtures, largely by charcoal adsorption and ionophoresis on silica gel. The resulting groups of peptides were then fractionated by paper chromatographic techniques into highly purified components that were partially characterized by qualitative end group and amino acid analyses. The presence of single residues of glycine, alanine, and isoleucine aided in the integration of the data into four larger peptide fragments. Since the complete sequences of the glycyl chain could not be deduced from such acid hydrolysis data, further degradative studies were conducted with pepsin, chymotrypsin, and carboxypeptidase. The resulting degradation products, separated by paper chromatography and partially characterized, completely confirmed the data obtained by acid degradation and provided a basis for elucidation of the amino acid sequence of the entire glycyl chain:



Sanger, Thompson & Kitai (20) determined the location of the six amide groups in insulin by study of the ionophoretic mobility and amide content of the fragments obtained from incubation of the two chains of insulin with a mold protease and papain. Amide groups in the glycyl chain are associated with the glutamic acid residues in positions five and fifteen, and with both aspartic acid residues. In the phenylalanyl chain, asparagine was located in position three, and glutamine in four.

At this point, elucidation of the complete amino acid sequence of insulin required only the location of the three disulfide bridges, some of which must join together the two peptide chains of the hormone. Initial attempts were frustrated by a random disulfide interchange reaction that occurred during acid hydrolysis of insulin, and produced a dismaying array of cystine-containing peptides that were not consistent with the previous data [Ryle & Sanger (21)]. However, the use of proteolytic enzymes and conditions of acid hydrolysis that avoided disulfide interchange (sulfuric acid solutions containing thiol compounds) provided authentic cystine peptides that were fractionated by ionophoresis and were oxidized to cysteic acid peptides. After ionophoretic separation of the cysteic acid peptides and partial characterization, the locations of the disulfide links were deduced [Ryle *et al.* (15)]. An intrachain link was found between the cysteine residues in positions six and eleven of the glycyl chain, and a disulfide link connected the seventh positions of the two chains. The third disulfide bridge was located between the twentieth and nineteenth positions of the glycyl and phenylalanyl chains, respectively. The structure is summarized:



structures of the insulins isolated from various species demonstrate that a highly specific sequence in the disulfide ring is not a prerequisite for biological activity. The differences, however, are relatively minor since position ten is always occupied by a branched chain residue, valine or isoleucine, position nine by serine or glycine, and position eight by alanine or threonine. Species' differences within the disulfide ring were not observed for the pituitary hormones. Perhaps specific configurational aspects of the cyclic structure in insulin are not as vital to physiological function as they are in oxytocin and vasopressin. Another spatial difference between the intrachain bridges of the pancreatic hormone and the posterior pituitary hormones is the existence of a half-cystine residue in the insulin ring that provides part of the interchain bridge between the insulin chains.

Aside from these speculative aspects, the relation of activity to chemical structure of insulin has been studied experimentally, perhaps more extensively than for any other protein. Both chemical and enzymatic approaches have been made to the problem. In 1950 J. & H. Fraenkel-Conrat (24) chemically modified various functional groups in insulin and reported the effect of these changes on biological activity. Covering of most of the amino and aliphatic hydroxyl groups caused little loss of activity; similarly, substitution of some of the amide, guanidyl, phenolic, and imidazole functions was without effect on activity. However, rupture of the disulfide bridges, esterification of the carboxyl groups, or extensive modification of the phenolic and imidazole functions all destroyed activity. In an extension of these observations, Fraenkel-Conrat (25) reported that the treatment of insulin with N-carboxyleucine anhydride resulted in extensive substitution of the  $\alpha$ - and  $\epsilon$ -amino functions, with no loss in activity. Mills (26) studied the substitution of the amino and phenolic functions of insulin with 2,4,5-trinitrotoluene, and confirmed the conclusion of Fraenkel-Conrat that the free amino groups were not necessary for biological function. Although the data indicated that extensive substitution of the amino groups destroyed activity, Mills postulated that the large size of the masking group sterically hindered biological function. Additional evidence that free  $\alpha$ -amino groups are not necessary for activity came from the preparation by Kaiser *et al.* (27) of biologically active phenyl- and allyl-thiocarbamyl derivatives of insulin. Recently Li (28) reported the reaction of 2,4-dinitrobenzene sulfonate with insulin, producing a selective substitution of the  $\epsilon$ -amino groups of the lysine residue. The derivative retained biological potency.

Information concerning structure-activity relationships of insulin has also been obtained by enzymatic degradation of the molecule. Early work had shown that insulin activity was destroyed by incubation with high concentrations of pepsin, trypsin, papain, and chymotrypsin. Later evidence [Harris (29); Harris & Li (30); Van Abeele & Campbell (31)] indicated that limited digestion with carboxypeptidase and with lower concentrations of trypsin liberated primarily a C-terminal alanine residue, with no loss in

biological potency. More extensive degradation with carboxypeptidase resulted in the partial loss of C-terminal asparagine, with accompanying destruction of activity; small quantities of tyrosine, glutamic acid, and leucine were also released, presumably from the glycyl chain of insulin following the removal of the C-terminal asparagine residue. Apparently the enzyme split peptide bonds on both sides of the penultimate half-cystine residue of the A chain, leaving this half of the cystine residue attached only by the disulfide link from the nineteen position of the B chain.

After prolonged tryptic digestion a heptapeptide product was liberated along with free alanine; loss of biological activity appeared to occur simultaneously with the release of the peptide. Thus, tryptic digestion resulted in a net loss of the entire C-terminal octapeptide sequence of the B chain.

Treatment of insulin under various conditions in concentrated urea solutions did not destroy biological activity; in some instances a delayed response was observed [Bischoff & Bakhtiar (32); Waugh *et al.* (33)]. Presumably, urea in high concentration effects an unfolding of the peptide chains. If such is the case, either insulin molecules can refold in a highly specific manner, or a completely rigid secondary structure is not vital for physiological function. Evidence that at least a portion of the insulin monomer exists in a rather rigid, preferred structure was presented by Hvidt & Linderstrøm-Lang (34), using the deuterium-exchange technique. Their data were interpreted as showing that stable internal hydrogen bridges exist between the  $\text{—CO—}$  and  $\text{—NH—}$  functions of the peptide bonds, providing a helical configuration. All readily exchangeable hydrogens are presumably present in the side chains and the end groups, not in the backbone. Linderstrøm-Lang (35) presented the view that insulin in aqueous solution contains stable hydrogen bonding only in the area of the "stabilized" portion of the molecule within the interchain disulfide bridges. However, there appeared to be a greater mobility of the elements in the intrachain disulfide ring structure, which was pictured as a loop outside the primary helical structure. The fact that all species differences occur in this intrachain ring structure may be explained by the concept of accessibility [Harris, Sanger & Naughton (23)]. In view of the presence of the interchain disulfide bridges in insulin, with the resulting stabilizing effects on the secondary structure of the hormone, elucidation of a specific structure-activity relationship may prove to be exceedingly difficult.

*Three-dimensional structures.*—Evidence based on crystallographic data has been interpreted as indicating that the insulin molecule (mol. wt. 12,000) could be represented by four closely packed rodlike structures, each of which forms a corner of a parallelogram [Low (36, 37)]. Each rod was presumed to represent a folded or coiled polypeptide chain located approximately 10 Å distant (from center to center) from another chain.

Hodgkin & Oughton (38) presented a possible configuration of insulin based, not on  $\alpha$ -helical considerations, but on fundamental considerations

of the geometry of the disulfide bridges, particularly the intrachain link. In their representation, the A and B chains of insulin were arranged in separate folded sheets, with the interchain cysteinyl residues extending from the same side of the sheets, and so arranged in space as to be linked in the appropriate disulfide bridges. While evidence against this  $\beta$ -type of representation exists, nevertheless it is of interest since the intrachain disulfide loop is easily accommodated.

Lindley & Rollett (39) constructed a model based largely on the  $\alpha$ -helix, with a modification imposed by the intrachain disulfide loop. On each side of position nine in the loop the  $\alpha$ -helix is wound in opposite directions, left-handed at the N-terminus, and right-handed in the C-terminal portion. The B chain appears to fit best a right-handed  $\alpha$ -helix, considered in light of the disulfide attachment to the A chain. The resulting two-chain model can readily be associated with a second similar unit to form a close-packed dimer that fits the unit cell dimensions imposed by crystallographic evidence. Based on present indications that the dimer is probably formed from two monomers by virtue of the linkage of one atom of zinc between two histidine residues [Tanford & Epstein (14)], Lindley & Rollett proposed that the linkage involves only the histidine in position ten of chain B. Waugh (40), drawing on his experiments with insulin fibrils, suggested that important factors in the stabilization of the insulin dimer and its association products were the position and occurrence of the non-polar side chains. In addition, the ionic effects of the side chain of tyrosine may also be important in this process. These two views on the stabilization of the insulin dimer differ considerably; however, it is possible that both factors may contribute to this phenomenon.

Linderstrøm-Lang (35), interpreting the data on deuterium exchange in an insulin monomer, and from previously reported suggestive evidence [Arndt & Riley (41)] for the presence of  $\alpha$ -helices in insulin, proposed that the insulin monomer is composed of two helices; the B and A chains were considered to exist as left- and right-handed helices, respectively, with the exception of the intrachain disulfide loop, which cannot be accommodated in an undistorted  $\alpha$ -helix. In solution, primarily those sections of the A and B chains within the stabilizing interchain disulfide bridges were thought to retain the specific helical configuration; most of the head and tail portions as well as the intrachain loop may exist in equilibrium between a folded and an unfolded state, with preference for the latter. Linderstrøm-Lang recognized that these speculations were based on deuterium exchange in an insulin monomer; dimerization or higher association is known to occur under the conditions of the experiments (pH 3), and may have affected the exchange results. However, some presumptive evidence from ribonuclease experiments suggests that association may not invalidate the data.

Great strides have been made in the past five years toward an understanding of the three-dimensional structure of insulin, although no mutually



exclusive representation has been described. The occurrence of disulfide bridges in insulin has been a complicating factor. Similar efforts directed toward a simpler crystalline substance, such as glucagon, may make available the three-dimensional structure of a small protein or polypeptide.

**Fibrils.**—Consideration of the mechanism of formation of insulin fibrils is of fundamental interest since present evidence indicates that the structure of the insulin molecule in the fibril remains essentially unchanged [Waugh (40)]. The formation of natural fibers may possibly occur in a similar manner. When aqueous insulin solutions below pH 3.5 are heated, a spontaneous molecular aggregation occurs, producing biologically inactive fibrils that are in a much more stable association form. Fibrils thus formed, seeded into a cold acid solution of native insulin provide a nucleus for quantitative conversion of all insulin in the cold solution to fibrils. Fibrils are stable in aqueous solution at pH values up to 10; above pH 10 soluble active insulin is regenerated. Waugh considers that the basic subunit of insulin that associates into fibrils is of mol. wt. 12,000, since under the conditions of the experiments the unit of mol. wt. 6000 is not observed. Modification of insulin by acetylation, esterification, and coupling with various diazonium salts does not prevent fibril formation, nor do solutions of concentrated acids or 6 *M* urea at pH 7.0 [Waugh *et al.* (33)]; an intact disulfide bridge system is necessary, however.

These data have been interpreted by Waugh (40) to indicate that covalent, electrostatic, and hydrogen bonding do not contribute significantly to the formation of fibrils. The hypothesis is presented that the large nonpolar side chains, representing 43 per cent of the side chains in insulin, are responsible for the type of linkage observed in fibril formation. A potential interaction energy of about 200 kcal. per mole of insulin is theoretically available from such side chains. Ionization of the phenolic functions above pH 10 may possibly be involved in the loss of stability of the fibril. Schematically, fibril formation appears to consist mainly of end to end binding of the units of mol. wt. 12,000 with a lateral staggered binding of additional units. Electron microscopic observation that the smallest fibrils are of the order of 50 to 80 Å in diameter [Farrant & Mercer (42)] suggests that several monomer chains are linked laterally.

**Inactivation.**—The question of the *in vivo* inactivation of insulin is one of potentially great practical significance as well as one of fundamental importance. Insulin is inactivated in the body by at least three different systems: an enzymatic system found in liver and other tissues, an immunochemical system in the blood of insulin-treated patients, and a chemical system dependent on reduction of disulfide bonds.

Since the first report in 1949 of an insulin-degrading enzyme in liver [Mirsky & Broh-Kahn (43)], interest in the degradation system has grown markedly. Insulin is degraded *in vitro* by slices or homogenates of a wide variety of tissues, and by plasma [Williams (44)]. Much effort has been directed toward purification of the liver-enzyme system. Evidence to date



suggests that liver contains both a heat-labile and a heat-stable degrading factor [Mirsky & Perisutti (45); Narahara *et al.* (46)]. Apparently the heat-stable factor is a reducing agent, such as glutathione, that inactivates insulin by rupture of disulfide bonds. Some evidence is available that the heat-labile factor inactivates insulin by proteolytic means [Vaughan (47); Tomizawa *et al.* (48)]. Mirsky, Perisutti & Dixon (49), for the sake of convenience, suggested that the enzyme be termed insulinase. In view of the fact that other substrates are attacked by the enzyme [Mirsky, Perisutti & Dixon (49); Tomizawa & Williams (50)], the term insulinase is perhaps inappropriate insofar as it suggests an enzyme specific for insulin degradation. A limited degree of specificity is associated with insulinase [Mirsky & Perisutti (51)], however, and use of the term in this sense is probably justifiable. Location of the specific bonds involved in the inactivation of insulin by the enzyme is presently being investigated. Results of these studies should be relevant to structure-activity considerations as well as to characterization of the mode of action of the enzyme.

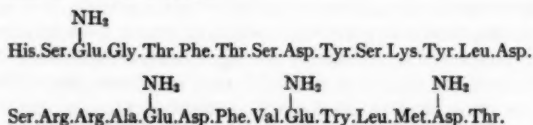
Despite the intensive efforts of several laboratories, the liver enzyme has not been obtained in homogeneous form. Recently Lewis & Thiele (52) described the isolation from elastase of a pancreatic insulinase preparation that appeared to be homogeneous during electrophoresis and ultracentrifugation. Further characterization of the enzyme, its mode of action, and its relationship to insulinase from other tissues must precede critical evaluation of the pancreatic enzyme.

Considerable evidence has accumulated that certain globulin-like factors, present in the blood of insulin-treated diabetics, bind insulin [Berson & Yalow (53)]. The reaction is immunological, involving an acquired antibody. Sera from normal individuals or from diabetic patients that have not received insulin do not bind insulin. The binding capacity of the sera from insulin-treated subjects rarely exceeds 0.01 unit per ml.; however, in rare cases of insulin resistance, the capacity may exceed 0.5 unit per ml. Berson & Yalow (53) have reported that the insulin-antibody complex migrates between the  $\gamma$ - and  $\alpha$ -globulins. Binding in most diabetic patients cannot be shown to be correlated with increased insulin resistance. In insulin-resistant patients, correlation of antibody content with resistance appears to be clear.

Interest in factors that inactivate insulin continues unabated and may well lead to a better understanding of protein structure as well as to information about the etiology and control of diabetes.

#### GLUCAGON

*Isolation.*—Interest in glucagon has increased markedly during the past decade. Attempts to obtain preparations of high purity were handicapped by difficulties encountered in separating glucagon from insulin. Such difficulties appeared to support the view that the two hormones are either similar chemically or may be derived during purification from a similar protein



Glucagon differs from insulin in virtually all structural aspects; only a few dipeptide sequences are similar. Another major difference between the hormones involves the lack of disulfide bridges in glucagon.

#### OXYTOCIN AND VASOPRESSIN

*Isolation.*—Just prior to 1952 du Vigneaud and his colleagues reported the isolation of beef oxytocin and vasopressin in nearly homogeneous form [Livermore & du Vigneaud (62); Turner, Pierce & du Vigneaud (63)]. In both instances countercurrent distribution was employed as the final preparative procedure as well as a major criterion of purity. Oxytocin was distributed between 2-butanol:0.05 per cent acetic acid ( $K=0.4$ ); vasopressin was purified using the countercurrent system, 1-butanol:0.09 *M* *p*-toluenesulfonic acid ( $K=1.25$ ). Later Pierce, Gordon & du Vigneaud (64) provided evidence from countercurrent studies that their beef oxytocin preparation was homogeneous. Preparations were subjected to 1000 transfers in the system described above; no appreciable fractionation occurred, and the distribution of the oxytocic material was in close agreement with theory. The crystalline flavianic acid salt of oxytocin had the expected potency and amino acid composition.

Additional evidence for homogeneity was provided by subjecting oxytocin preparations to zone electrophoresis and to column chromatography [Kunkel, Taylor & du Vigneaud (65); Taylor (66); Condliffe (67)]. From the electrophoretic data the isoelectric point was determined to be about pH 7.7. Pierce, Gordon & du Vigneaud (64) also reported the isolation of oxytocin from porcine glands using the same methods that had been employed for the preparation of beef oxytocin; the two preparations were identical in all respects.

Such similarity of the hormones from different species was not found for vasopressin. Highly purified pressor preparations from porcine pituitaries exhibited a markedly different distribution coefficient ( $K=0.66$ ) from beef vasopressin [Popenoe, Lawler & du Vigneaud (68)]. The separate identity and the homogeneity of these two pressor hormones were further established by zone electrophoresis and column chromatography [Taylor, du Vigneaud & Kunkel (69); Taylor (66); Condliffe (67)]. Electrophoretic analysis confirmed previous data that the isoelectric point of vasopressin is at pH 10.9. Fromageot & colleagues (70, 71) and Tuppy (72), using somewhat different preparative procedures, also isolated vasopressin and oxytocin, respectively, of sufficient purity for structural analysis.

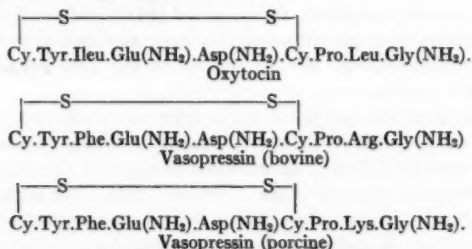
Ward & du Vigneaud (73) recently published an improved method of isolation of hog vasopressin using conventional extraction methods followed by zone electrophoretic fractionation and countercurrent distribution.

*Structural analysis.*—The amino acid composition of oxytocin and vasopressin from bovine glands had been well established prior to 1952. Each hormone contains three amide groups and equimolar quantities of eight amino acid residues, six of which appear in both hormones: tyrosine, proline,

glutamic acid, aspartic acid, glycine, and cystine. In addition oxytocin contains leucine and isoleucine, and vasopressin contains arginine and phenylalanine. Pierce, Gordon & du Vigneaud (64) demonstrated that hog oxytocin is identical in amino acid composition with the bovine hormone; Popenoe, Lawler & du Vigneaud (68) found that porcine vasopressin contains lysine in place of the arginine that occurs in the beef hormone.

These pure preparations of extremely potent well-characterized hormones of small molecular weight served as model polypeptides, ideally suited for structural analysis and possible synthesis. Interest in the structure of the posterior pituitary hormones during 1950 to 1953 culminated in a dramatic climax during June to September, 1953. In this brief period three groups submitted reports describing the complete amino acid sequence of both oxytocin and vasopressin. Investigations in du Vigneaud's laboratory led to the elucidation of the structures of both hormones [du Vigneaud, Ressler & Trippett (74); du Vigneaud, Lawler & Popenoe (75)]. Tuppy (72) and the collaborators of Fromageot (76) independently announced the structures of oxytocin and vasopressin, respectively.

The peptide hormones, either before or after oxidation with performic acid, were submitted to partial acid degradation or enzymatic digestion. The resulting fragments were isolated, largely by paper and column chromatography, or ionophoresis, and were partially characterized. The complete amino acid sequences were deduced from the unequivocal arrangement of the degradation products, giving the structures:



Although no direct evidence was available that glutamic and aspartic acids were present as amide derivatives, this deduction by all three groups of investigators was later confirmed by the work of Lawler *et al.* (77) who demonstrated that asparagine and glutamine were released from oxytocin and vasopressin by the action of papain. Synthesis of the hormones provided unequivocal evidence that the structures deduced from degradative work were correct.

*Synthesis.*—The report describing the first synthesis of a polypeptide hormone, namely oxytocin, was presented by du Vigneaud *et al.* (78) simultaneously with the paper describing the structure of the hormone. Details of the synthesis were reported later by du Vigneaud *et al.* (79). Announcements of the synthesis of arginine- and lysine-vasopressin appeared from the same

laboratory [du Vigneaud, Gish & Katsoyannis (80); Bartlett *et al.* (81)]. A detailed report of the synthesis of lysine-vasopressin was recently published by du Vigneaud, Bartlett & Jöhl (82). In each case the final steps included reduction of the protected linear S,S'-dibenzylnonapeptide to the sulphydryl nonapeptide, and mild oxidation in air to close the disulfide link. The synthetic oxytocin and vasopressins exhibited the expected spectrum of biological activities in the proper ratios. Furthermore, the synthetic products were indistinguishable from the natural hormones under a variety of conditions, including countercurrent distribution, electrophoresis, quantitative amino acid composition, and infrared spectrum. One significant difference between the natural and synthetic hormones was observed: the pressor activity of the purified synthetic vasopressins was lower than that of the natural hormones. Some inactivation of arginine vasopressin occurred during the final lyophilization of the product. The unequivocal proof of identity of the natural and synthetic vasopressins awaits the preparation of pure synthetic hormones. However, it can be concluded that the structures of these posterior lobe hormones are established beyond reasonable doubt. A more comprehensive treatment of the isolation, structure, and synthesis of the hormones may be found in reviews by du Vigneaud (83, 84).

*Structure-activity relationships.*—The isolation and synthesis of oxytocin and vasopressin have made possible a clarification of the contribution of each hormone to the various biological activities that were associated with crude extracts of the posterior pituitary gland. These activities include milk ejection, avian vasodepression, uterine contraction, antidiuresis, and pressor effects. Despite previous reports to the contrary, both oxytocin and vasopressin exhibit all of these biological activities, but to markedly differing extents [du Vigneaud (83); Gyermek & Fekete (85)]. Unquestionably, oxytocin is primarily responsible for the vasodepressing, the uterine-contracting, and the milk-ejecting [Whittlestone (86); Cross & Van Dyke (87)] effects. Pure oxytocin contains about 500 USP units of each activity per mg.

In addition, homogeneous ox and pig vasopressin possess uterine-contracting, milk-ejecting, and avian-vasodepressing effects to the extent of 5 per cent, 20 per cent, and 15 per cent, respectively, of the activity found in pure oxytocin [Popenoe *et al.* (88); Lawler & du Vigneaud (89); Van Dyke, Engel & Adamsons (90)].

Vasopressin is clearly the major pressor and antidiuretic principle of the posterior pituitary, with potencies of each activity in the order of 600 USP units per mg. Nevertheless, pure natural (or synthetic) oxytocin also exhibits antidiuretic and pressor activities, although only to about 0.5 per cent and 1 per cent, respectively, of the activity associated with pure vasopressin [Van Dyke, Adamsons & Engel (91); Gyermek & Fekete (85)].

Van Dyke, Engel & Adamsons (90) found that lysine-vasopressin is identical in biological activity with arginine-vasopressin with one minor exception: the lysine-containing hormone exhibits only one-seventh the antidiuretic potency of the arginine analogue when assayed intravenously

in dogs. When the hormones were assayed subcutaneously in rats, the anti-diuretic potencies were essentially the same. The authors tentatively concluded on the basis of such activity measurements, using pituitary extracts from other species, that lysine-vasopressin is peculiar to hogs and that the vasopressins of man, monkey, dog, sheep, and camel are identical with arginine-vasopressin. Proof for this interesting suggestion will require the isolation and characterization of the hormone from each of these species.

In view of the close similarity in chemical structure of oxytocin and vasopressin, the partial sharing of biological function by the hormones does not seem illogical. Indeed, the marked biological change that accompanies the substitution of phenylalanine for isoleucine and arginine for leucine provides another example of the great specificity that is often observed in biological systems.

The opportunities for altering the chemical structures of oxytocin and vasopressin are manifold; however, the unequivocal evaluation of the chemical change in terms of biological activity is somewhat more difficult. Perhaps the most striking aspect of the chemistry of the two hormones is the nearly identical 20-membered disulfide ring structure common to both peptides. Ryle *et al.* (15) demonstrated that insulin also contains a disulfide ring of identical size. From the standpoint of hormonal function, such structures are of obvious interest. The importance of the specific cyclic structure is emphasized by the finding of Ressler & du Vigneaud (92) that the isoglutamine isomer of oxytocin exhibits none of the expected biological activities. The principal structural change thus effected in the molecule is the introduction of two methylene units in the ring moiety, producing a 22-membered ring. Presumably, the configuration of the molecule as governed by the disulfide ring is of vital concern to biological activity.

Ressler (93) synthesized the cyclic pentapeptide(amide) ring moiety of oxytocin; the molecule possessed significant oxytocic and milk-ejecting potency, 3 and 1 units per mg., respectively, but no measurable depressor, pressor, or antidiuretic activity. The disulfide of the linear heptapeptide, Ileu. Glu. Asp. Cys. Pro. Leu. Gly(NH<sub>2</sub>), was completely devoid of all activity.

The weak oxytocic activity in the cyclic ring structure is of great interest. Apparently an important function of the side chain moiety is in potentiating the activity. Similar studies with the ring moiety of vasopressin may be anticipated with interest, for they will complement the oxytocin studies.

Additional information on these points was provided by the synthesis of oxypressin, a substance having the cyclic pentapeptide structure of the vasopressins and the tripeptide side chain of oxytocin [Katsoyannis (94)]. This synthetic hybrid possessed both oxytocic and vasopressor activities, although to a much lesser degree than the pure hormones. Oxypressin exhibited about 5 per cent and 10 per cent, respectively, of the oxytocic and depressor activity of pure vasopressin. The ratio of the activities as well as the greater



oxytocic activity suggests the possibility that the presence of arginine or lysine in the side chain may be particularly vital for pressor activity. The information is in accord with the speculation that the intact 20-membered disulfide ring structure is basic for activity, but the type of biological activity and the intensity of the activity may be governed by the influence of the side chain.

Lawler & du Vigneaud (89), and du Vigneaud, Lawler & Popenoe (75) found that tryptic digestion of beef and pig vasopressin resulted in the liberation of glycineamide with the simultaneous loss of all biological potency. This finding is in accord with the established specificity of trypsin; similarly the resistance of oxytocin to the enzyme may have been expected. The loss of both the oxytocic and the pressor activities of vasopressin is of interest. This may indicate that the intact leucylglycineamide sequence in oxytocin is a prerequisite for activity, or perhaps the C-terminal residue must be in the amide form. Again, the importance of the side chain to biological activity is demonstrated.

The correlation of biological activity and chemical structure is obviously not simple, even in comparatively simple peptide hormones. To date the facts point to the requirement of some interrelations of the tripeptide side chains and the 20-membered disulfide rings. Additional experimental work now in progress may help in the understanding of this fascinating problem.

*Single posterior pituitary hormone?*—Early speculation that the various activities found in crude posterior pituitary extracts were associated with a single protein hormone received some support from biological and chemical studies. The isolation of pure oxytocin and vasopressin does not preclude such a hypothesis since these small polypeptides could have been liberated during the isolation, e.g. during the initial extraction of the gland with hot glacial acetic acid.

The best chemical evidence for the existence of a single hormone stemmed from the work of Van Dyke *et al.* (95), wherein a preparation of 30,000 mol. wt. having both oxytocic and pressor activities (1:1 ratio) was isolated under mild conditions from posterior lobes. The preparation appeared homogeneous according to ultracentrifugal and solubility studies; electrophoretic examination indicated a high degree of purity.

Recent work of Acher *et al.* (96, 97) provided evidence that the protein of Van Dyke was inadequately characterized, since dialysis, electro dialysis, trichloroacetic acid precipitation, and countercurrent distribution all effected separation of hormonal activity and inert protein. Countercurrent distribution was conducted in the solvent, 2-butanol:0.5 per cent trichloroacetic acid. The data showed a separation of oxytocic and vasopressor activities from the bulk of the original protein; vasopressin and oxytocin, examined in the same partition system, possessed distribution coefficients identical with the respective activities associated with the Van Dyke protein. Furthermore, the isolated active materials were indistinguishable from oxytocin and vasopressin on paper chromatographic examination. Similar separations were



obtained when the Van Dyke protein was subjected to electrodialysis. The isolated active preparation migrated in a fashion identical with oxytocin and vasopressin, and possessed physiological activities and amino acid compositions indistinguishable from the pure hormones. These data clearly refute the hypothesis that the Van Dyke protein represents the single posterior lobe hormone, and provide an additional example of the need for cautious evaluation of data pertaining to the homogeneity of proteins. Presently available chemical evidence, then, indicates that the hormones are distinct chemical entities and provides no evidence of a single origin.

#### CORTICOTROPIN

*Isolation.*—Corticotropin has been the object of intensive research in the past several years, culminating in the independent announcement by three separate groups of the isolation and the structural analysis of the hormone from two species. Despite the publication of several reports describing the isolation of corticotropin, including the recent comprehensive review of Li (98), this aspect of the subject will be considered briefly because of its possible relevance to the situation existing presently for somatotropin, prolactin, thyrotropin, and other biologically active proteins of higher molecular weight.

As late as 1952 reports appeared which verified the apparent homogeneity of corticotropin, a protein with a molecular weight of approximately 20,000 [cf. Li & Pedersen (99)]. Evidence for the homogeneity of such preparations had been obtained in various laboratories by conventional electrophoretic, diffusion, ultracentrifugation, and solubility studies. The inadequacy of such criteria of homogeneity was clear when reports were published describing the fractionation of "pure" corticotropin by means of electrodialysis, paper electrophoresis, countercurrent distribution, ion exchange, and adsorption chromatography. Such fractionations led to the isolation of pure preparations of corticotropin having a molecular weight of about 4500. Quite obviously, the older conventional methods for demonstration of homogeneity were inadequate when applied to this protein.

All three groups who obtained pure corticotropin employed adaptations of the elegant procedures introduced by Astwood and his colleagues (100, 101, 102), which yielded preparations of approximately 25 to 90 USP units per mg. Bell (103) and Shepherd *et al.* (104) reported the preparation of homogeneous  $\beta$ -corticotropin using countercurrent distribution with the system, 3.5 per cent NaCl in 6 per cent acetic acid:1-butanol. Seven other equally active corticotropins were observed but extensive characterization was confined to the  $\beta$ -moiety, which was present to the largest extent (*ca.* 50 per cent). White & Landmann (105) fractionated oxycellulose eluates on columns of Amberlite XE-97 in 0.1 *M* sodium carbonate-bicarbonate buffer at pH 8.5, followed by repeated countercurrent distribution of the more active fraction in the solvent, 2-butanol:0.2 per cent trichloroacetic acid.

Corticotropin-A was thus prepared in about 93 per cent purity, with little or no indication of the presence of other active moieties. Li and co-workers (106, 107) isolated  $\alpha$ -corticotropin from sheep pituitaries by precipitating impurities from the oxycellulose-purified material with dioxane and subjecting the resulting supernatant to zone electrophoresis on starch at pH 11.1. The active fraction from electrophoresis was further fractionated on columns of Amberlite XE-97 and by countercurrent distribution in the system, 2-butanol:0.5 per cent trichloroacetic acid. In addition to the pure  $\alpha$ -corticotropin prepared in this manner Li and co-workers also isolated a second component,  $\alpha_2$ -corticotropin, that contained two more amide groups. Evidence was presented that  $\alpha_2$  was converted to  $\alpha$  by the alkaline conditions employed during certain isolation steps.

The use of countercurrent distribution as a final purification step and as an important criterion of purity is particularly noteworthy. This technique undoubtedly is of tremendous value in the isolation and characterization of polypeptides, although the inherently complicated chemical nature of peptides does not in every case allow an unequivocal interpretation of such solvent distributions. The development and application of newer, more sensitive fractionation techniques radically altered the course of research with the corticotropins. Whether such a situation exists in the case of other proteins, e.g., growth hormone, remains to be seen.

The preparative methods employed by the three groups of investigators, while not identical, nevertheless bear considerable resemblance. For example, each employed a relatively harsh initial extraction, an oxycellulose adsorption-elution, and finally, countercurrent distribution. Only during the isolation of  $\beta$ -corticotropin by Shepherd *et al.* (104) were numerous other hormonally-active corticotropins observed. The other active moieties might conceivably have been formed during the extensive countercurrent distribution procedure; indeed evidence was presented that  $\beta_4$ -corticotropin originated in this manner. However, the other isolated components, including the  $\beta$ -fraction, had reproducible distribution coefficients, suggesting that no protein interactions occurred during the process. Furthermore, pure  $\beta$ -corticotropin remained unchanged after a four-hour incubation at 70°C. in glacial acetic acid, indicating that the most severe of the purification steps employed by Shepherd *et al.* did not produce other fractions from  $\beta$ .

Thus the several active moieties of corticotropin are quite probably present in the gland. All available information indicates that the chemical differences between the active fractions are small; this emphasizes the remarkably high resolving power of the countercurrent system used in the isolation. The isolation of several components by Shepherd *et al.* probably was dependent on the availability of oxycellulose eluates of high specific activity (90 USP units per mg.) and the quantitative recovery of individual components from countercurrent distributions. Only about 10 per cent of the oxycellulose product was inert protein, whereas Li *et al.* (106) and White

& Fierce (108) obtained oxycellulose products of the order of 25 USP units per mg., or containing 75 to 85 per cent of inactive constituents. In the subsequent purification of these products of lower specific activity other active principles, present in very small quantities, may have been lost with the impurities. An alternative possibility is recognized, namely that the sheep, in contrast to the hog, may not elaborate several active moieties.

Further indication of additional active corticotropin components in pork glands was obtained during careful chromatographic analyses by Dixon & Stack-Dunne (109). Chromatography of oxycellulose concentrates on columns of finely ground Amberlite IRC 50 (XE-64) demonstrated the presence of at least three active components,  $A_1$ ,  $A_2$ , and  $A_3$ . The fraction  $A_2$  may arise from  $A_1$  by loss of amide ammonia; however, evidence was also presented that  $A_2$  exists as such in the gland.

*Peptic digestion.*—Li first presented evidence nearly ten years ago that the activity of corticotropin preparations was retained after partial peptic hydrolysis. Since the evidence then available indicated that corticotropin was a pure protein of mol. wt. 20,000, this knowledge stimulated considerable effort toward isolation of a small active peptic degradation product that might be amenable to synthesis. In 1952 Brink *et al.* (110) announced the preparation of a seemingly pure peptic digestion product of corticotropin, corticotropin-B. Again countercurrent distribution was used as the final purification step and as a major criterion of purity [Kuehl *et al.* (111)]. The material appeared to be approximately 95 per cent homogeneous after a 20 tube distribution in the solvent, 2-butanol:0.5 per cent trichloroacetic acid. The distribution coefficient was 0.5 to 0.6. Amino acid and ultracentrifugal analyses provided evidence for a molecular weight in the range of 5000 to 7000. As noted earlier, pure undigested corticotropin has a smaller molecular weight than that reported for this digested product. These observations appear to be in conflict and lend support to the suggestion that additional characterization of the digested product is needed.

Bell *et al.* (112) showed clearly that the incubation of pure  $\beta$ -corticotropin with pepsin resulted in degradation to three smaller active products. The importance of utilizing a pure substrate was indicated by Bell and co-workers; they suggested that peptic digestion of an oxycellulose preparation may lead to 25 to 30 different active products. Thus, emphasis is given to the difficulties encountered by Brink *et al.* (110) in the isolation of corticotropin-B and in its characterization.

*Structural analysis.*—Few if any proteins are as well characterized as corticotropin. Three groups, working independently, succeeded in establishing almost simultaneously the amino acid sequences of three different corticotropin preparations: (a) Corticotropin-A from pig glands [White (113); White & Landmann (105)], (b)  $\beta$ -corticotropin from pig glands [Bell (103); Howard *et al.* (114); Shepherd *et al.* (115)], and (c)  $\alpha$ -corticotropin from sheep glands [Li *et al.* (116)]. End group analyses demonstrated that all

three corticotropins were probably single-chain proteins containing N-terminal serine and C-terminal phenylalanine. Amino acid analyses indicated that each preparation was composed of 39 amino acid residues, the only difference being that the sheep hormone contained one more serine and one less leucine than the two pig preparations, which were identical in this respect. Structural analysis was accomplished by (a) enzymatic cleavage of the molecule with trypsin, pepsin, and chymotrypsin; (b) isolation of some or all of the degradation peptides by countercurrent distribution, paper chromatography, or paper electrophoresis; (c) characterization of the peptides by amino acid, end group, and step-wise sequential analyses using the Edman (117), carboxypeptidase and leucine aminopeptidase techniques; and (d) the unequivocal deduction of the total amino acid sequence from knowledge of the structures of the degradation products. The meticulous work of Shepherd, Bell and co-workers in which nearly quantitative recovery of the peptides was obtained from countercurrent distribution, may be cited as an outstanding example of protein structure analysis. Unquestionably very little, if any, microheterogeneity is possible in  $\beta$ -corticotropin. The sequence of amino acids was reported to be:

Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.Val.Gly.Lys.Lys.Arg.Pro.Val.  
Lys.Val.Tyr.Pro.Asp.Gly.Alu.Glu.Asp.Glu(NH<sub>2</sub>).Leu.Ala.Glu.Ala.Phe.Pro.Leu.Glu.Phe.

Such quantitative recovery and analysis is probably not necessary to establish the unique structure of a peptide or small protein. The amino acid sequences proposed for pig corticotropin-A and sheep  $\alpha$ -corticotropin were based on evidence somewhat less quantitative. Only relatively minor structural differences were reported for the three corticotropin molecules; these all occur in the sequences (25 to 32 inclusive) that are not necessary for activity:

	25	26	27	28	29	30	31	32
—	Asp.	Gly.	Ala.	Glu.	Asp.	Glu.	Leu.	Ala—
	$\beta$ -Corticotropin (pig)							
—	Gly.	Ala.	Glu.	Asp.	Glu.	Leu.	Ala—	
	Corticotropin—A (pig)							
—	Ala.	Gly.	Glu.	Asp.	Asp.	Glu.	Ala.	Ser—
	$\alpha$ -Corticotropin (sheep)							

The differences may be due to (a) the origin in two animal species, (b) the instability of sequence 25 to 28 during Edman degradation [Shepherd *et al.* (115)], and (c) the variations in preparative procedures.

Additional examples of structural analyses without recourse to extensive quantitative data are found in the elucidation of the amino acid sequences in insulin, oxytocin, vasopressin, and intermedin. The amino acid sequences of these hormones have been confirmed in essential aspects. Thus, there is reason for increasing confidence in the validity of structures deduced from entirely consistent sequential data that are obtained from protein degrada-

tion products liberated by acid treatment or by the action of two or more enzymes. For ultimate proof of structure the synthesis by unequivocal methods and the comparison of synthetic material with the natural product is desirable. Only with oxytocin and vasopressin have syntheses been achieved. Because of the size and complexity of other protein hormones the attainment of this goal in some cases is improbable. Thus far, the work of Hofmann and co-workers (118, 119, 120) in synthesizing portions of the pig corticotropin molecule has completely verified the structures proposed from degradative methods [e.g., White & Landmann (121)]. The major synthetic effort is being directed toward the N-terminus of the molecule, for this portion appears to be necessary for biological activity. The following peptides have been synthesized and have been found to be identical with natural enzymatic degradation products: Ser.Tyr, Arg.Try, Glu.Phe, and Ser.Tyr. Ser.Met.Glu. Many peptides that probably represent portions of the hormone but do not correspond to natural degradation products have been prepared by Hofmann and co-workers, including Glu.His.Phe.Arg. All of the synthetic peptides were carefully characterized by optical rotation studies and by enzymatic digestion with leucine aminopeptidase or carboxypeptidase followed by quantitative amino acid analysis. In all cases theoretical yields of the amino acids were obtained, demonstrating stereochemical homogeneity. The critical importance of configurational analysis in peptide synthesis is apparent, since racemization commonly occurs with many synthetic procedures.

*Beef corticotropin.*—The beef corticotropins isolated by White & Peters (122) and by Li & Dixon (123) appear to be identical in amino acid content and C- and N-terminal sequences with  $\alpha$ -corticotropin prepared from sheep glands. Reports describing the complete amino acid sequence are awaited with interest, as such information will permit definite conclusions as to the identity of beef and sheep corticotropin.

*Structure-activity relationships.*—Much of the information regarding the relation of corticotropin structure to biological activity has been obtained through the use of enzymatic degradations. Quantitative liberation of the three C-terminal residues, phenylalanine, glutamic acid, and leucine, by carboxypeptidase treatment did not affect biological activity [Harris & Li (124)]. One of the peptic digestion products of  $\beta$ -corticotropin that was isolated by Shepherd and co-workers (115) differed from the parent substance only in the loss of a C-terminal portion containing the last 11 residues. Mild acid hydrolysis appeared to liberate an additional four residues from the C-terminus without destroying biological activity.

White (125), using leucine aminopeptidase digestions, showed that one or both of the first two N-terminal residues are necessary for biological activity. Loss of approximately half of the N-terminal residue, serine, and the adjacent residue, tyrosine, resulted in a roughly comparable loss in activity. Extensive peptic degradation liberated, among other peptides, the

N-terminal peptide, Ser.Tyr.Ser.Met.Glu, with concomitant loss in activity [White & Landmann (126)]. White & Gross (127) recently reported that incubation of corticotropin-A with bovine fibrinolysin resulted in loss of biological activity. Fibrinolysin behaved like a selective trypsin in that only two major splits occurred, between residues 8 and 9, -Arg.Try-, and between residues 15 and 16, -Lys.Lys-. Clearly these data show that the intact N-terminal penta- or octapeptides of corticotropin per se do not have biological activity. The observations with fibrinolysin also correspond to those with trypsin in that the latter enzyme causes destruction of biological activity.

Shepherd *et al.* (104) reported that boiling  $\beta$ -corticotropin for 20 min. in 0.1 *N* NaOH caused a sharp drop in activity; partial loss of residues 1, 2 and 3 was observed. The alkali treatment greatly potentiated the low intermedin activity consistently associated with pure ACTH preparations; such data are in accord with previous findings with crude ACTH preparations. Sequences 7 through 13 in intermedin and 4 through 10 in ACTH are identical; apparently, then, the loss of the three N-terminal residues in ACTH served to destroy the corticotropin activity and release the latent MSH activity simultaneously. It would be of interest to determine the intermedin activity of various products of the partial digestion of ACTH with leucine aminopeptidase. Interestingly, pure intermedin preparations exhibit no ACTH activity. Although these two hormones do contain a common heptapeptide sequence, the isolation and structural analysis proves beyond a doubt that they are distinct chemical entities.

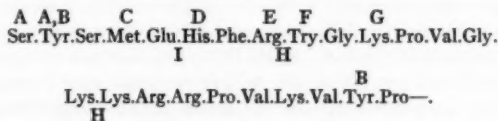
In a recent report Dedman, Farmer & Morris (128) confirmed and extended earlier observations that corticotropin is inactivated by oxidizing agents. The phenomenon is a puzzling one since this protein contains no detectable disulfide or sulfhydryl groups. Oxidation with hydrogen peroxide or ferricyanide markedly reduces the potency of the hormone, and subsequent treatment with thiol compounds completely reverses the inactivation. The oxidized form of ACTH can be separated from the native protein by column chromatography [Dixon & Stack-Dunne (109); Dixon (129, 130); Farmer & Morris (131)]. Incubation of ACTH with peroxydisulfate or periodate also destroys activity, but in an irreversible manner. Differences between the oxidation products formed by the two sets of reagents are not apparent. Dedman, Farmer & Morris (128) pointed out that only five residues in the essential portion of the ACTH molecule may be expected to be involved in the reversible oxidation-reduction phenomenon. These are serine, methionine, tyrosine, tryptophan and histidine. All are single residues with the exception of the two residues of serine. Since chemical destruction of methionine and partial photochemical destruction (78 per cent) of histidine were not paralleled by a significant drop in biological activity, these residues do not appear to be involved. Attempts to detect the formation of a carbonyl group from oxidation of the alcohol side chain of serine were unsuccessful.



ful, and the serine content remained unchanged after oxidation; hence serine is probably not the redox-sensitive center. The complete iodination of most of the tyrosine residues gave a partially active product that was still sensitive to oxidation; reduction of the oxidized form yielded a product with biological activity intermediate between that of the original and that of the iodinated protein. This evidence, coupled with the unchanged ultraviolet spectrum and the unchanged tyrosine content after oxidation made it appear unlikely that tyrosine was involved. The possible role of tryptophan in the reversible inactivation was investigated. No apparent difference was observed between the condensation products formed from oxidized and native ACTH with *p*-dimethylaminobenzaldehyde although in both cases activity was destroyed. The condensation presumably occurs on the 2-position of the indole ring. The oxidized ACTH contained virtually the same tryptophan content and did not differ in ultraviolet absorption characteristics from the untreated ACTH. Thus, it was concluded that tryptophan was probably not an important factor in the reversible oxidation-reduction. Dedman and co-workers suggested that the peculiar oxidation-reduction behavior of ACTH may be attributable to a "hitherto unidentified group." A knowledge of this phenomenon is certainly basic to the understanding of structure-activity relationships.

Carr, Conn & Wartman (132) reported that ACTH binds zinc and copper ions in acid solution. Other substances that possess a similar property include salmine and dihydrostreptomycin; common features of all three substances are their cationic properties in acid and their high content of guanidine groups. Under certain conditions a relationship exists between corticotropin activity and the extent of zinc binding. Although arginine alone does not bind zinc under these conditions the guanidine group of arginine, in conjunction with other structural features, may be important to the maintenance of biological activity.

Some of the information regarding the relationship of the chemical structure of corticotropin to biological activity may be summarized as follows, using the 24-residue fragment observed by Shepherd *et al.* (115) as the smallest known active corticotropin:



A—One or both of the N-terminal serine and tyrosine are necessary for activity.

B—Partial inactivation occurs when tyrosines are heavily iodinated.

C—Methionine can be desulfurized without loss of activity.

D—78 per cent of histidine can be photo-oxidized without loss of activity.

E—Guanidine group may be related to the capacity to bind metals and other ions; integrity of the group appears to be correlated with activity.

F—2-position may be necessary for activity.

G—Free amino groups needed for activity.

H—Split by fibrinolysin, with complete loss of activity.

I—Split by pepsin with loss of activity.



The absence of a rigid, stable secondary structure in corticotropin is made probable by the demonstration by Linderstrøm-Lang (133) that ACTH in solution readily exchanges all hydrogen atoms that can be expected to exchange with deuterium. Similar results were obtained for simple peptides, but not for proteins such as insulin. Whereas these data appear to preclude a stable secondary structure for corticotropin in solution, the molecules may assume a preferred, more stable configuration at the site of action. Since the presence of residues 1 and 2 is necessary for activity, but penta- or octapeptides containing these residues are completely inactive, some configurational considerations are probably important in the understanding of the relationship of ACTH structure to biological activity.

In summary, the isolation and characterization studies with corticotropin illustrate some points of fundamental interest in the chemistry of peptides and proteins. The need for extensive and discriminating characterization of a protein preparation is amply documented. Application of newer criteria of purity such as zone electrophoresis, countercurrent distribution, end-group analysis, and column chromatography, as well as conventional solubility, electrophoresis, and ultracentrifuge studies is indicated. The nearly quantitative recovery of the degradation products and the careful sequential analysis of  $\beta$ -corticotropin probably provide the best available evidence against covalent microheterogeneity. However, the same painstaking work provides some evidence that chemically different moieties of the protein hormone may be present in the pituitary gland. To this extent a "limited microheterogeneity" probably does exist. Finally, the well-documented but mysterious oxidation-reduction behavior of corticotropin may be taken as indication that the secrets of structure-activity relationships may go deeper than merely a unique arrangement of amino acid residues, or a specific configuration of the peptide chain, or both. The laborious synthetic work now in progress may ultimately provide the key to the solution of the many perplexing questions regarding the relationship of chemical structure to biological activity.

#### GROWTH HORMONE

*Isolation.*—Two methods (134, 135) have been available for the preparation of apparently homogeneous crystalline bovine growth hormone. In 1954 Ellis *et al.* (136) modified the isolation procedure, and shortly thereafter Li (137) provided a simplified method based on salting-out and isoelectric precipitations that gave a yield of 2 gm. of hormone preparation per kg. of ox anterior pituitary tissue. Growth hormone has also been prepared from sheep, pig, horse, fish, monkey, and human glands (138, 139, 140), although the bovine preparation has been most extensively characterized. Comprehensive reviews dealing with growth hormone may be found in the volume, *Hypophyseal Growth Hormone, Nature and Actions* (141), and in a more recent paper by Li (98).

*Homogeneity.*—Earlier work had provided much evidence from electrophoresis, ultracentrifuge, and solubility determinations that bovine prep-

arations were of high purity. However, the availability of newer methods has led to a reappraisal of the conclusions.

Pierce (142) subjected the hormone to a countercurrent partition in a 2-butanol:*p*-toluenesulfonic acid solution; despite the limited number of transfers employed and the high partition coefficient exhibited by the protein hormone under these conditions, some indication was obtained that the better preparations were reasonably homogeneous. Fønss-Bech & Li (143), using starch zone electrophoresis at pH 4, 9, and 12.2, demonstrated that the protein migrated as a single zone in all cases. Using Hyflo-Supercel as the adsorbent in column chromatography Clauser & Li (144) reported that the protein hormone was represented by a single peak in the effluent. In each of the above characterizations the peak representing the active protein material was not symmetrical, but within the limits of bioassay no differences in specific activity were detected in the leading and trailing edges of the peak material.

However, Ellis and co-workers (136), subjecting various bovine preparations to electrophoresis in 0.015 ionic strength buffers at pH 4.0 and 9.6, reported that growth hormone prepared by the Ellis *et al.* (136), Wilhelm (135), or Li (137) methods was heterogeneous. In addition, solubility data were presented that suggested heterogeneity. Ellis & Simpson (145) found that the purified preparations responded to fractionation on carboxymethylcellulose columns, and that non-growth-promoting impurities, amounting to approximately 28 per cent of the original sample, were removed. A nonlinear gradient elution using 1 *M* NaCl flowing into 0.03 *M* acetate buffer (ionic strength, 0.03; pH 5.0), was employed. In columns of diethylaminoethylcellulose, buffered at pH 8.7, use of a combined pH and salt gradient led to separation of electrophoretically homogeneous growth hormone into two components. Both materials retained full biological potency. Rechromatography of the individual components suggested that the two protein substances were inter-convertible. Such a phenomenon may be similar to that previously reported by Li & Papkoff (146). These workers had demonstrated electrophoretically that purified bovine growth hormone, under relatively mild conditions, was converted to a substance that migrated 1.3 times faster at pH 4.0. Since full activity was retained under certain conditions, apparently both substances were equally potent. Raben (147) chromatographed purified growth hormone over carboxymethylcellulose columns in 2.8 *M* acetic acid solution with an increasing gradient of  $\text{CaCl}_2$  from 0.001 *M* to 0.02 *M*. Under these relatively rigorous conditions two major components were partially separated, and Raben stated that the growth-promoting activity was concentrated in one component. Since it was reported that little, if any, growth hormone was lost on the column, presumably a large fraction of the starting material was inert protein.

N-terminal analysis of highly purified bovine growth hormone has been reported by Reid (148), Li & Ash (149), and Levy & Li (150). Alanine and

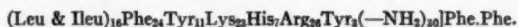
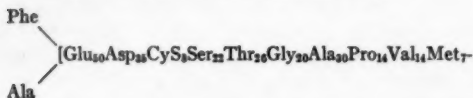
phenylalanine, in ratios of one mole each per mole of hormone (assumed mol. wt. 47,000), were the only N-terminal residues observed by the dinitrophenylation and phenylthiohydantoin methods. The presence of certain impurities might have escaped detection; these include substances that have no N-terminal residues, that have the same N-terminal residues as growth hormone, or that contained certain amino acids in an amino-terminal position that are extremely difficult to determine quantitatively. Nevertheless the chemical data is good supplementary evidence for the homogeneity of bovine growth hormone.

Critical discussion of the homogeneity of various preparations of beef growth hormone is obviously difficult at the present time. A complicating factor is that growth hormone is known to be convertible, under rather mild conditions, to a different substance; thus, the ability to fractionate growth hormone preparations into two components does not necessarily prove gross heterogeneity, but, instead, may depend upon the conditions of the experiment. Certainly the body of evidence as a whole, particularly in conjunction with the clear-cut N-terminal analyses, indicates that purified bovine growth hormone represents a reasonably homogeneous protein. However, papers describing possible heterogeneity should not be disregarded; the situation with bovine growth hormone may be analogous to that previously described for corticotropin.

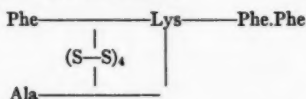
**Denaturation.**—The appearance of an electrophoretically faster moving component in bovine growth hormone solutions during various manipulations is an interesting phenomenon. Under some conditions, i.e., 0.1 *N* NaOH at 25°C. for 72 min., 50 per cent of the protein is converted to the fast fraction with full retention of potency. In 0.1 *M* acetic acid at 25°C. for 360 min. a similar amount of conversion takes place, but the product is inactive (146). Incubation of growth hormone with chymotrypsin at 0°C. for 30 min. also led to formation of a fast moving fraction; no proteolytic splitting was detected [Li *et al.* (151)]. Probably the fast moving component is a product of denaturation of growth hormone; formation occurs in the presence of water, possibly influenced by temperature or the presence of an enzyme (98). Since the denatured substance may or may not retain biological potency, depending on the conditions, Li (98) suggested that different forms of denatured growth hormone exist, as, for example, an active and inactive form. While it is assumed that both forms may arise from native protein, and that the active form of denatured protein is convertible into the inactive form, the reactions postulated are irreversible.

**Structure.**—Bovine growth hormone was subjected to C-terminal analysis using carboxypeptidase [Harris *et al.* (152)],  $\text{LiAlH}_4$  reduction [Jutisz (153)], and hydrazinolysis [Geschwind (154)]. Treatment with  $\text{LiAlH}_4$  and hydrazine yielded quantitative data that phenylalanine was the sole C-terminal amino acid. Since carboxypeptidase liberated two moles of phenylalanine per 45,000 gm. of growth hormone, the C-terminal sequence of the

hormone must be . . . Phe.Phe. Knowledge of the end-groups and the amino acid composition [Li & Chung (155)] of bovine growth hormone makes possible the following summary:



Since oxidative cleavage of the bovine hormone with performic acid resulted in the conversion of all four cystine residues to cysteic acid without apparently splitting the molecule into two or more fragments, Li (98) proposed the following tentative structure:



**Structure-activity relations.**—Since no prosthetic group has been detected in growth hormone, studies have been made to ascertain which functional groups are necessary for biological activity. Apparently the integrity of certain of the tyrosine side chains [Li, Simpson & Evans (156)] and the  $\epsilon$ -amino groups of lysine [Reid (148)] is essential to the maintenance of full biological potency.

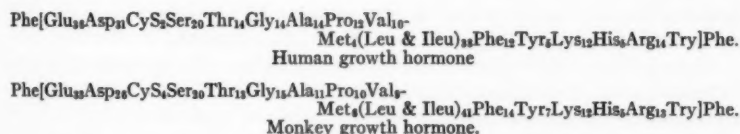
Growth hormone is obviously a well-studied protein, but the complete characterization of the amino acid sequence and chemical structure of this molecule of 45,000 mol. wt. represents a formidable task. For this reason it is pertinent to note the experiments of Harris *et al.* (152) and Li and co-workers (151, 157) in which growth hormone was degraded under certain conditions with either carboxypeptidase, trypsin, or chymotrypsin with complete retention of growth-promoting ability. The treatment with chymotrypsin or trypsin caused splitting of about 30 per cent of the peptide bonds and may have led to the formation of a relatively small active fragment. The feasibility of structural studies depends upon the molecular size of the active fragment(s), and the ability to prepare it in a homogeneous form. The active material(s) from tryptic and chymotryptic digestion are not dialyzable, an indication that they may still be of considerable size and complexity. Furthermore, such digestion with proteolytic enzymes may give rise to numerous active moieties that may be extremely difficult to separate.

**Species differences.**—Repeated failure to demonstrate significant effects of bovine growth hormone in man has stimulated work regarding species differences. For example, Pickford (158) found that the growth hormones isolated from fish and ox glands were equally effective in fish, although the fish hormone appears to be inactive in mammals [Wilhelmi (138)]. Knobil

& Greep (159) showed that monkeys do not respond to the bovine hormone. Wilhelmi (138) described some chemical and physical differences among the hormones prepared from ox, sheep, pig, and fish glands, thus providing a partial basis for understanding the observed species differences in physiological response.

As an approach to the study of growth hormone action in man, much effort has been directed recently toward isolation and characterization of human and monkey hormone. Li & Papkoff (139) and Raben (140) isolated growth hormone from the pituitaries of both species, and Beck *et al.* (160) reported that both hormone preparations as isolated by Raben were effective in man.

Li & Papkoff (139) and Li (161) reported comparisons of the chemical and physical properties of bovine, monkey, and human hormones that were homogeneous according to electrophoresis and ultracentrifuge studies. All appeared to be different; the human and monkey hormones—each having a molecular weight of approximately 25,000—differed considerably from the bovine preparation of mol. wt. 46,000. Studies with dinitrofluorobenzene and carboxypeptidase provided information that the human and monkey hormones contain phenylalanine in both the N- and C-terminal positions. These data along with the amino acid analyses permit the following formulations:



Despite the observed chemical and biological differences among the hormones, complete species specificity is clearly lacking; e.g., the bovine hormone is active in fish and rats, and the monkey and human hormones are effective in rats. Reasoning from these facts, Wilhelmi (138) and Li (161) suggested the possibility that the growth hormones contain a common core that is responsible for the hormonal function. Some experimental support for this concept has already been obtained by Li and co-workers (151, 152, 157), since they found that a considerable portion of the bovine hormone can be removed without loss of biological activity.

#### MELANOCYTE-STIMULATING HORMONE

*Isolation.*—Interest in the isolation of the melanocyte-stimulating hormone (MSH) or intermedin was recently stimulated, both directly and indirectly, by the research that led to the isolation and structural analysis of ACTH. The preparative steps developed for the isolation of crude ACTH were found to be adaptable to the isolation of MSH. Furthermore, the crude preparations of ACTH were found to be more potent in melanocyte-stimu-

lating activity than MSH preparations previously reported. A great interest was stimulated in determining whether ACTH and MSH activities were present in the same protein molecule. The resulting intensive studies of MSH culminated in reports from two laboratories, appearing only weeks apart, of the complete amino acid sequence of the  $\beta$ -melanocyte-stimulating hormone isolated from pig glands [Harris & Roos (162); Geschwind, Li & Barnafi (163)].

Landgrebe & Mitchell (164) reported that melanocyte-stimulating activity was readily concentrated by use of ACTH purification techniques. Modification of the method of Astwood and his colleagues (100, 101, 102) by use of extraction with hot glacial acetic acid, oxycellulose adsorption-elution, and precipitation of the activity in the cold by about 80 per cent acetone at pH 6.5, permitted 20 to 30 per cent yields of pig MSH preparations as potent as 950 I.U. per mg.

During the first five months of 1955 three separate reports were submitted for publication describing swine MSH preparations that essentially satisfied at least one criterion of purity [Lerner & Lee (165); Benfey & Purvis (166); Porath *et al.* (167)]. All three groups employed glacial acetic acid-extracted and oxycellulose-treated hog pituitary preparations as a starting material for further purification. Lerner & Lee employed the lyophilized oxycellulose eluate directly, while the other two groups used material derived from the oxycellulose step and precipitated in the cold by about 80 per cent acetone at pH 6.5 [cf. Landgrebe & Mitchell (164)].

Lerner & Lee (165, 168) distributed the oxycellulose concentrate through 12 transfers in the countercurrent system, 2-butanol:0.5 per cent trichloroacetic acid. The material of high MSH activity in tubes three to five was subjected to further fractionation by paper electrophoresis at pH 8.9 in barbiturate buffer. Re-electrophoresis of the most potent component at pH 4.9 in pyridine-acetate buffer indicated the presence of a single active product. Additional electrophoresis under a variety of conditions, and countercurrent distribution (97 transfers) in the above solvent system provided evidence for homogeneity of the order of 95 per cent. The final product assayed approximately 1000 to 2000 I.U.<sup>4</sup> per mg.; no vasopressin or ACTH activity was found in experiments designed to detect one unit of each. The authors point out that the instability of highly purified intermedin in solution supports the suggestion that the pure hormone may assay somewhat higher. The isoelectric point was estimated to be in the pH range 10.5 to 11.0, based on paper electrophoresis observations. Amino acid analysis

<sup>4</sup> For the sake of admittedly crude comparisons, all potencies for intermedin are expressed in terms of International Units (I.U.); many American workers prefer the intermedin assay and unitage as defined by Shizume, K., Lerner, A. B., and Fitzpatrick, T. B., *Endocrinology*, **54**, 553 (1954). One I.U. is approximately 10<sup>4</sup> Shizume units [cf. Geschwind, I. I., and Li, C. H., *J. Am. Chem. Soc.*, **79**, 615 (1957)].



indicated that swine MSH is composed of about 37 amino acid residues and has a minimum molecular weight of about 4500.

Benfey & Purvis (166, 169) prepared a highly purified MSH by countercurrent distribution in 2-butanol:0.5 per cent trichloroacetic acid of acetone-precipitated oxycellulose eluates. The final product, virtually free of ACTH activity and assaying 2500 I.U. per mg., was 80 to 85 per cent pure according to analytical countercurrent distribution studies. When material from the peak tubes was subjected to amino acid analysis, evidence was obtained that pig intermedin contained 30 amino acid residues, differing qualitatively as well as quantitatively from the preparation of Lerner & Lee (165, 168). The minimum molecular weight as calculated from the amino acid data was approximately 3800.

Porath *et al.* (167), using the starting material previously described [Landgrebe & Mitchell (164)], obtained a highly purified MSH preparation by means of zone electrophoresis on starch in a vertical column with pH 4.8, 0.1 *M* pyridinium acetate buffer. A second electrophoretic fractionation under identical conditions yielded an MSH preparation that was homogeneous according to ultracentrifugal and electrophoretic studies. The homogeneous product assayed about 1500 I.U. per mg. Similarity to the preparation of Lerner & Lee ended at this point since the isoelectric point was found to be 5.2, the molecular weight was estimated at about 3000, and the qualitative amino acid analysis differed considerably. Porath *et al.* (167) suggested the possibility that Lerner & Lee had isolated a product of aggregation of intermedin with a basic component present in the oxycellulose eluate.

The considerable confusion regarding the actual characteristics of swine MSH appeared to be dispelled by the later publication of Lee & Lerner (168). Since over-all yields of MSH in their isolation procedure were only about 5 to 15 per cent, Lee & Lerner sought evidence that their preparation represented the main active MSH in the gland. Countercurrent distribution in 2-butanol:0.5 per cent trichloroacetic acid of crude posterior pituitary powder from hogs yielded two biologically active components. The major one had a distribution coefficient (2.1) identical with that of their earlier preparation and was called  $\alpha$ -MSH; the minor component with a distribution coefficient of 0.6 was designated  $\beta$ -MSH.  $\alpha$ -MSH accounted for approximately 75 per cent of the total recovered MSH activity. Similar countercurrent distribution of either the oxycellulose concentrate or the preliminary countercurrent distribution product also yielded two components with distribution coefficients identical with those of  $\alpha$ - and  $\beta$ -MSH. Lee & Lerner had apparently disregarded the  $\beta$ -MSH during the original paper electrophoretic fractionation and had isolated only  $\alpha$ -MSH.

Application of the same countercurrent system to crude MSH, prepared according to the method of Landgrebe & Mitchell, yielded a single active component that closely resembled  $\beta$ -MSH. Since Benfey & Purvis and Porath *et al.* had utilized the preparative scheme of Landgrebe & Mitchell,



apparently these workers also had isolated only  $\beta$ -MSH. Lerner & Lee noted that the precipitation step employing 80 per cent acetone at pH 6.5 (Landgrebe & Mitchell) may not precipitate a protein such as  $\alpha$ -MSH with an isoelectric point of 10.5 to 11.0. Investigating this possibility, they were able to identify relatively large quantities of  $\alpha$ -MSH in the 80 per cent acetone supernatant.

$\alpha$ -MSH and  $\beta$ -MSH probably exist as such in the gland and are not artifacts resulting from the preparative procedures. This statement is supported by the reproducible distribution coefficients for the two components and by their probable presence in acetone-dried glands.

The countercurrent distribution data of Lerner & Lee which differentiated  $\alpha$ - and  $\beta$ -MSH were obtained using biological assay as the analytical tool. Crude materials were employed, and it is conceivable that the impurities may have altered the distribution curves. Nevertheless, the excellent reproducibility and the logical agreement of all data provide excellent evidence that the major MSH component of pig glands is  $\alpha$ -MSH, as isolated by Lerner & Lee, and the minor component is  $\beta$ -MSH, as isolated by Benfey & Purvis and Porath *et al.*

Geschwind, Li & Barnafi (163) and Geschwind & Li (170) also isolated a pure porcine MSH; later work proved its identity with the  $\beta$ -MSH obtained by Porath *et al.* The method of purification is only slightly different from that of other investigators up to and including adsorption of the MSH on oxycellulose. Elution from the oxycellulose was accomplished with 0.1 *N* HCl, the solution was neutralized with methyldioctylamine, and impurities (largely ACTH) were precipitated at pH 6.5 to 7.0. In light of the data of Lee & Lerner, such a product would be expected to contain both  $\alpha$ - and  $\beta$ -MSH; crude posterior pituitary powders such as those used as starting material contained both moieties, preliminary purification methods were quite similar, and the cold 80 per cent acetone precipitation method was not used. However, throughout the remainder of the isolation scheme, Geschwind & Li showed that the majority of the MSH activity was due to the component that emerged as  $\beta$ -MSH after repeated starch zone electrophoresis and countercurrent distributions. No adequate explanation is available for this apparent discrepancy between the data from the two laboratories.

It can be concluded that two active MSH moieties appear to be present in pig pituitary glands, somewhat analogous to the situation with corticotropin wherein several chemically different active ACTH substances were found. In view of the structural and biological similarities between ACTH and MSH, knowledge of the structure of  $\alpha$ -MSH would indeed be of interest.

Benfey & Purvis (169) also described the isolation of highly purified MSH from beef glands. They subjected preparations purified by Stehle (171) to countercurrent distributions in the system 2-butanol:0.1 per cent trichloroacetic acid. Redistribution of the most active material through 24

transfers provided indication that the preparation was approaching homogeneity, although no estimate of purity was given by the authors. Material isolated from the peak tubes ( $K=0.74-0.85$ ) contained 47 amino acid residues and had a molecular weight of approximately 5600. The assay of 450 I.U. per mg. sharply differentiates the material from hog MSH. Another marked difference was the threefold increase in potency caused by alkaline incubation, a property not shared by  $\alpha$ - or  $\beta$ -MSH. Such potentiation may be compared with the effect of alkali on  $\beta$ -corticotropin.

Recently, Geschwind, Li & Barnafi (172) published a preliminary report describing the isolation and structural analysis of a bovine MSH. Essentially the same isolation scheme was employed as that used for  $\beta$ -MSH. The isolated product differed somewhat from  $\beta$ -MSH, although by no means as much as suggested by the data of Benfey & Purvis (169). The bovine MSH exhibited a greater cathodic mobility, a lower distribution coefficient in the solvent 2-butanol:0.5 per cent trichloroacetic acid and a higher isoelectric point (pH 7) than  $\beta$ -MSH. The amino acid composition was identical with that of  $\beta$ -MSH with the exception that bovine MSH contained one more residue of serine and one less of glutamic acid.

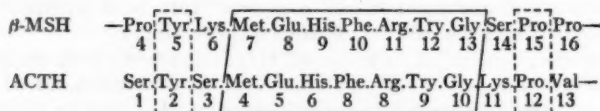
The bovine MSH isolated by Benfey & Purvis (169) clearly differs from that prepared by Geschwind, Li & Barnafi (172). The initial isolation schemes differ considerably, and it is entirely conceivable that these two groups of workers have isolated two different bovine melanocyte-stimulating hormones, as was the case with  $\alpha$ - and  $\beta$ -MSH from pig glands. The behavior of the preparation of Benfey & Purvis during alkali incubation suggests the possibility that their preparation may be related to a form of ACTH; however, evidence supporting this suggestion is lacking since their bovine MSH bears little physical resemblance to any known ACTH and probably exhibits little, if any, ACTH activity.

*Structural considerations.*—Harris & Roos (162), in a preliminary report, described the complete amino acid sequence of  $\beta$ -MSH, as isolated by Porath *et al.* (167). The hormone was degraded enzymatically with trypsin and chymotrypsin. The peptide products of digestion by each enzyme were separated and isolated by paper ionophoresis, and their amino acid compositions were determined by paper chromatography. The intact hormone and selected degradation peptides were subjected to the step-wise Edman degradation, providing evidence for the unequivocal assignment of all amino acid sequences:

Asp.Glu.Gly.Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp.  
1 2 3 4 4 6 7 8 9 10 11 12 13 14 15 16 17 18

The sequence 7 to 13 is identical with that between 4 and 10 in ACTH and provides a logical basis for the inherent MSH activity of ACTH. Differences in other portions of the molecules prove conclusively that the two hormones are different.

Geschwind, Li & Barnafi (163, 173), using a somewhat similar degradative approach, completely confirmed the amino acid sequence of  $\beta$ -MSH published by Harris & Roos. Geschwind, Li & Barnafi point out that, aside from the interchange of lysine and serine at two points in the MSH and ACTH molecules, the following decapeptide sequence would be common to the two hormones:



This situation may be compared with that for oxytocin and vasopressin, polypeptides that differ at only two points in a nonapeptide sequence.

The common heptapeptide and surrounding sequences in ACTH and MSH provide a fertile field for speculation and for studies relating chemical structure to biological activity. Extensive synthetic work is already under way on the N-terminal portion of corticotropin; structures related to oxytocin and vasopressin are also being investigated. Such investigations may contribute to a basic understanding of some aspects of the relationship of hormonal activity to peptide structure.

#### PROLACTIN

*Isolation.*—Prolactin was obtained in highly purified form twenty years ago [Lyons (174); White, Catchpole & Long (175)]. Recent modification of the isolation procedure [Cole & Li (176)] makes available a relatively simple method of obtaining in good yields two gm. of apparently homogeneous prolactin from one kg. of sheep glands. The starting material is the acid acetone precipitate from a first step in the isolation of  $\alpha$ -corticotropin. Such material is salted out with sodium chloride, and is further purified by an isoelectric precipitation which yields a prolactin preparation of about 20 I.U. per mg. Fifteen countercurrent transfers in the system 2-butanol:0.4 per cent dichloroacetic acid, followed by an additional isoelectric precipitation, yielded a preparation assaying 35 I.U. per mg. Countercurrent distribution in the same solvent system provided evidence that this material is a single component.

*Homogeneity.*—In addition to the evidence for homogeneity afforded by the countercurrent distribution, previous electrophoretic and solubility studies had indicated that highly purified prolactin preparations were homogeneous [Li, Lyons & Evans (177, 178)]. Similarly, when Cole, Geschwind & Li (179) applied quantitative N-terminal analyses to highly purified preparations, threonine was found to be the sole N-terminal residue. Using the dinitrophenylation, phenylthiocarbamyl, and periodate methods, one mole of DNP-threonine, threonine phenylthiohydantoin, and acetaldehyde, respectively, were found per mole of prolactin (molecular weight assumed to

be 25,000). While studies with column chromatographic and zone electrophoretic techniques are lacking, the evidence to date provides a strong case for the homogeneity of prolactin preparations.

*Structural analysis.*—In an extension of the previously mentioned N-terminal analysis of prolactin by use of the paper strip phenylthiocarbamyl method, Cole, Geschwind & Li (179) reported nearly quantitative yields of the phenylthiohydantoins of threonine, proline, valine, threonine, and proline, in that order.

Li (180) had published earlier the complete amino acid composition of prolactin using chemical and microbiological analyses. These data indicated that the minimum molecular weight of prolactin is 33,000, which is in reasonable agreement with some physicochemical determinations, notably sedimentation-diffusion studies. Subsequently Li (181) published preliminary results of an amino acid analysis of a prolactin preparation that was homogeneous from the standpoint of countercurrent distribution and N-terminal analysis. The analysis utilized the method of Levy (182) and led to a calculated molecular weight of about 25,000. The data are in good agreement with previous osmotic pressure and diffusion and viscosity studies, as well as with the N-terminal analyses. Using the more recent amino acid data of Li, it is possible to summarize the evidence concerning the chemical structure of prolactin as follows:

Thr.Pro.Val.Thr.Pro[Arg<sub>10</sub>Asp<sub>25</sub>Cys<sub>3</sub>Glu<sub>13</sub>Gly<sub>13</sub>His<sub>7</sub>(Ileu & Leu)<sub>13</sub>  
Lys<sub>10</sub>Met<sub>7</sub>Phe<sub>3</sub>Pro<sub>15</sub>Ser<sub>13</sub>Thr<sub>7</sub>Tyr<sub>7</sub>Try<sub>1</sub>(-NH<sub>2</sub>)<sub>12</sub>Val<sub>11</sub>]

#### THYROTROPIN

*Isolation.*—Prior to 1952 the techniques used in the isolation and purification of thyrotropin from beef or swine pituitary glands were largely the classical protein precipitation methods [e.g., Ciereszko (183); Fraenkel-Conrat *et al.* (184)]. Fels *et al.* (185) in 1952 published a modified method in which 1.5 gm. of purified material, virtually free of contaminating hormones, was obtained per kg. of beef anterior lobes. The method included extraction with dilute acetic acid-saline solutions, ammonium sulfate fractionation, and cold acetone precipitation. Further modification and extension of this method was recently reported by Fels, Simpson & Evans (186). More notable alterations include an initial alkaline extraction, oxycellulose adsorption of impurities, and a sodium chloride trichloroacetic acid precipitation. Careful testing of the final product, assaying about two to three USP units per mg., indicated the virtual absence of other known hormones. Other recent purification attempts have applied the newer preparative methods to products that were partially purified by well-established means [cf Ciereszko (183)]. Thus, Steelman *et al.* (187, 188) subjected thyrotropin concentrates to repeated fractional electrical transport at 7,000 v. 10 m. amp. for 25 hr. and obtained in small amounts a preparation which assayed 10 USP units per mg. From these data the isoelectric point of thyrotropin

was estimated to be in the pH range 8.0 to 8.5. Critical evaluation of the data is difficult since detailed experiments have not been described, nor have attempts to duplicate the work been published. Since some success was achieved by use of a separation technique based largely on charge differences, application of the newer preparative zone electrophoretic methods may be profitable.

Most of the recent efforts to purify thyrotropin have relied upon column chromatographic separations. Heideman (189), using an acetone and ammonium sulfate-fractionated starting material, accomplished about a fifteenfold purification on columns of Amberlite IRC-50 buffered at pH 8.0. After washing away protein impurities with water, the active hormone was eluted with either 1 *M* NaCl, HCl, or  $\text{CaCl}_2$  solutions. The cold eluates were treated with picric acid or trichloroacetic acid (approximately 7 per cent) to remove additional impurities, and the active product was isolated from the supernatant by dialysis and lyophilization. Attempts to chromatograph thyrotropin preparations at a pH higher than 8 failed since the activity was not adsorbed. The observation is consistent with the suggestion of Steelman *et al.* (187) that the isoelectric point is in the pH range, 8 to 9.

Crigler & Waugh (190), in a preliminary note, report that preparations comparable in potency to those of Fels, Simpson & Evans (186) may be obtained simply by Amberlite IRC-50 chromatography of a water extract of acetone powder of beef anterior pituitaries. The columns were buffered at pH 7.6 with 0.01 *M* phosphate buffer and the active material was eluted with 1 *M* sodium chloride. Rechromatography was reported further to enhance the specific activity, and an indication was given that purification was successfully achieved in a batch-wise manner. Details of the work were not given.

Pierce & Nyc (191) started with a thyrotropin concentrate of 0.8 USP units per mg. prepared by the method of Ciereszko (183), and achieved a sevenfold purification on columns of Amberlite IRC-50 (XE-64) resin. Thyrotropin was chromatographed initially on columns buffered with 0.2 *M* phosphate at pH 6.45, followed by a second fractionation on similar columns at pH 5.95 using an increasing concentration gradient for elution. Condliffe & Bates (192), using a similar starting material, essentially confirmed the results of Pierce & Nyc (191) with XE-64, and extended the chromatography of thyrotropin to include the adsorbent carboxymethylcellulose. The thyrotropin activity was eluted from carboxymethylcellulose columns at pH 6.0 with increasing sodium ion concentration. With the nearly quantitative recovery of activity from such columns, about 1.6 gm. of thyrotropin assaying 5 USP units per mg. may be recovered from each kg. of bovine anterior pituitary powder.

Further purification of thyrotropin was announced recently in a preliminary communication by Condliffe & Bates (193). The products from carboxymethylcellulose columns were fractionated on columns of diethyl-

aminoethylcellulose using an increasing ionic strength gradient from 0.005 to 0.2 in pH 9.5 glycine buffer. Several milligrams of thyrotropin which assayed about 15 USP units per mg. were recovered, representing the most potent preparation attained to date. The authors report that thyrotropin, in the low concentrations required for assay, is unstable, suggesting that their preparation may be more potent than reported.

*Characterization.*—Material prepared by the method of Ciereszko had previously been reported to be homogeneous on the basis of electrophoretic and ultracentrifugal data [White (194)]. Comparison of the specific activity of preparations prior to the establishment of a standard in 1952 is difficult. It seems likely that the recent preparations assaying as much as 15 USP units per mg., obtained from column chromatographic and from electrical transport methods, are more potent than those obtained prior to 1952 with precipitation methods. The chromatographic studies furthermore suggest that inhomogeneity exists, even in the purer preparations. Such purified preparations were reported by Pierce & Nyc (191) and Condliffe & Bates (192) to have sedimentation constants of approximately 3.0. These data contrast sharply with the reports of White (194) and Fels, Simpson & Evans (186) that their preparations, under similar sedimentation conditions, have a sedimentation constant of 1.0, with an apparent molecular weight of about 6,000 to 10,000. Recognition should be taken of the fact that Fels, Simpson & Evans (186) demonstrated with analytical paper electrophoresis and Solka-Floc column chromatography that their preparations were not homogeneous. The relative potency of the active material isolated from Solka-Floc fractionation was not reported. Apparently, the more highly purified preparations have a higher molecular weight.

The highly active thyrotropin preparations reported recently still contain some carbohydrate material [Hays & Steelman (188)]. Free amino end groups were not detectable in two different thyrotropin preparations, using the dinitrophenylation method [Hays & Steelman (188); Fels, Simpson & Evans (186)]; this suggests the possibility that (a) this site is occupied by carbohydrate, or (b) this portion of the peptide chain is circular, or (c) certain amino acids difficult to detect (e.g., proline, serine, glycine, and hydroxyproline) may occupy the N-terminus. Analysis of the C-terminal residues with carboxypeptidase and hydrazinolysis and reinvestigation of the N-terminus with leucine aminopeptidase and the Edman degradation are certainly in order.

Speculations concerning the nature of the thyrotropin molecule have little meaning when considerable doubt exists as to the homogeneity of the preparations. It would appear that the hormone has a molecular weight considerably greater than 10,000, and may be a mucoprotein containing no N-terminal residues. The possibility of obtaining a smaller active molecule is suggested by the finding of Fels *et al.* (185) that papain-digested thyrotropin preparations contain hormonally-active material that dialyzes and



is not precipitated in 20 per cent trichloroacetic acid. No attempts to isolate and characterize such active fragments have been recorded, possibly because of a low yield of activity or the presence of a multiplicity of active moieties. Investigation of such active thyrotropin fragments prepared from digestion with papain or other more specific proteolytic enzymes may permit more definite conclusions as to the molecular nature of the hormone.

#### INTERSTITIAL CELL-STIMULATING HORMONE

Since the announcements in 1940 that homogeneous preparations of pig and sheep interstitial cell-stimulating hormone (ICSH) had been prepared [Li, Simpson & Evans (195); Shedlovsky *et al.* (196)], interest has apparently been diverted from this gonadotropic hormone to other pituitary hormones. Few additional chemical characterization or homogeneity studies of these preparations have been reported. The pig and sheep hormones differed widely in chemical properties, having molecular weights of 100,000 and 40,000 respectively. Undoubtedly such preparations should be reexamined for homogeneity with the more recent methods of protein fractionation.

Although earlier reports indicated that beef pituitary was a relatively poor source of ICSH, Takeda *et al.* (197) isolated and crystallized the hormone from acetone powders of beef anterior pituitaries. The preparation behaved as a single component electrophoretically. A more recent publication by this group [Otsuka & Noda (198)] provided a modified method of isolation which yielded a crystalline preparation twice as potent as the first crystalline material. Acetone powders of anterior pituitary tissue were extracted with the solvent, methanol:pH 5.0 acetate buffer (4:6), for 24 hr. The ICSH activity was precipitated by increasing the methanol concentration to 85 per cent. The precipitate was dissolved in water, and impurities were precipitated with 2-ethoxy-6,9-diaminoacridinium lactate. Addition of 10 volumes of acetone precipitated the ICSH. The active fraction was redissolved in water and was purified further by removal of an inactive precipitate that formed in the presence of 30 per cent acetone. Crystallization occurred when the acetone concentration was increased to 50 per cent and the solution was cooled. Some indication was obtained that the presence of metal ions was necessary for crystallization of the mucoprotein. The beef preparations contained 5.9 per cent mannose and 6.1 per cent glucosamine, indicating a closer chemical resemblance to sheep ICSH than to the pig hormone.

#### FOLLICLE-STIMULATING HORMONE

*Isolation.*—Earlier work had shown that follicle-stimulating hormone (FSH) may be isolated from human, horse, pig, sheep, and beef anterior pituitary glands. Most of the recent purification attempts have employed either pig or sheep sources; glands from these species are intermediate in



FSH potency between the relatively rich source—horse, and the poor source—beef.

Since FSH appears to be the only pituitary hormone that is soluble in concentrated salt solutions, e.g., 50 per cent ammonium sulfate, virtually all initial isolation methods have been based upon this property. Li & Pedersen (199) observed that sheep FSH, prepared by repeated ammonium sulfate and alcohol fractionation [Li, Simpson & Evans (200)], behaved as a single component in electrophoresis, diffusion, and ultracentrifugation. Preparations of swine FSH, nearly comparable in potency to the sheep preparation of Li and co-workers, have been reported by Van Dyke, P'an & Shedlovsky (201) and by Steelman *et al.* (202). Purification was accomplished similarly by repeated salt, alcohol, and isoelectric precipitations. Swine and sheep FSH appear to be similar chemically but may be differentiated immunologically [Van Dyke, P'an & Shedlovsky (201)].

In 1954 McShan, Kagawa & Meyer (203) presented a simplified method of preparation of sheep FSH from acetone-dried pituitary powder. The powder was extracted at 72°C. with saturated sodium chloride solutions, and after precipitation of contaminating ICSH at pH 2.5, 30°C., the FSH activity was recovered by batchwise adsorption-elution with the anion exchange resin, Amberlite XE-59. Such preparations, although highly potent and relatively free from other hormones, were not electrophoretically homogeneous. Further purification was achieved by chromatography of the preparations on columns of the cation exchange resin, Amberlite XE-97 [McShan & Meyer (204)]. After fractionation at pH 6 in acetate solutions, additional purification was achieved by rechromatography at pH 5.0. Lack of a uniform assay or common standard prevents critical comparison of the various preparations; however, McShan & Meyer implied that their product from resin chromatography was probably superior to previously reported FSH preparations in being as potent in FSH activity but less contaminated with ICSH activity.

Stelman, Lamont & Baltes (205, 206) reported that attempts to purify FSH further by means of classical protein precipitation methods were unsuccessful; they were led to try other approaches to the problem. Pancreatin-digested FSH preparations responded to fractionation with alcohol, yielding FSH about ten times as potent as previous preparations. In addition, undigested preparations subjected to separation by fractional electrical transport yielded small quantities of FSH, at least five times as potent as preparations obtained solely by classical precipitation methods. The role of pancreatin in the purification is not clear; a change in the FSH molecule may be involved. Additional purification of the pancreatin-digested product was recently reported in a preliminary note [Stelman *et al.* (207)]. Repeated chromatography with columns of diethylaminoethylcellulose at pH 7.0, low ionic strength, and gradient elution in 0.5 M NaCl-0.1 M Na<sub>2</sub>HPO<sub>4</sub> solutions led to recovery of an apparently homogeneous product. The

theoretical elution curve and the results of study with paper electrophoresis and the ultracentrifuge supported this conclusion. The biological potency was approximately 30 to 50 times that achieved by precipitation methods.

*Characterization.*—The preparative method of Steelman *et al.* should be confirmed and more extensive criteria of purity should be applied to the final FSH preparation before a definitive statement can be made that this preparation of 29,000 mol. wt. represents a homogeneous FSH.

The purified product was similar to other preparations in that it contained carbohydrate. Total sugar content was seven to eight per cent; about one-half was hexosamine, and the remainder was mannose, galactose, and fucose. The importance of the carbohydrate moiety of the molecule is indicated by the earlier observations that FSH activity was lost during incubation with *ptyalin* and *takadiastase*.

Additional studies will be required to determine whether the pancreatin treatment produces a change in the FSH molecule. Most proteins undergo change under the influence of the numerous enzymes that are present in pancreatin. Assuming that some bonds in FSH have been ruptured, an analogy with the ACTH-pepsin system suggests that other active FSH molecules may be found in the digest.

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# BIOCHEMISTRY OF VIRUSES<sup>1</sup>

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The last review of the biochemistry of viruses to appear in this series was that of Putnam (1) in 1956. The present reviewer will discuss the newer work on the structure of viruses and the function of their components, in so far as they can be understood by the use of biochemical techniques. The biological studies which border on the topic of virus biochemistry, especially the field of virus genetics, will not be considered in great detail, since several reviews of these topics have been published recently (2, 3, 4). The possibilities for chemotherapeutic treatment of virus diseases will not be discussed as such, since an excellent review has been written by Matthews & Smith (5) and Horsfall & Tamm (308). The material is arranged according to type of virus as follows: I. Plant viruses, II. Insect viruses, III. Animal viruses, and IV. Bacterial viruses.

## SPHERICAL PLANT VIRUSES

Spherical plant viruses are approximately isodiametric plant viruses containing RNA, most of which have been obtained in crystalline form. Turnip yellow mosaic virus is a typical representative of this group and one which has been well studied. It has an unusually high RNA content of 34 per cent, and the RNA is only loosely bound to the protein. According to Cohen & Schachman (6) the RNA is heterogeneous with a molecular weight of  $4.6 \times 10^4$ , or  $1 \times 10^6$  depending on the methods of preparation. This nucleic acid can be separated from the protein by treatment with 30 per cent ethanol. The nucleic acid-free protein is also formed in infected plants along with the infectious virus. This protein has a lower density than the virus and therefore can be separated from it by fractional centrifugation. The preparation of the two fractions has been refined by Cosentino *et al.* (7, 8), who have also published the results of comparative electron microscopy and preliminary chemical analyses. These workers have confirmed the concept proposed by Markham & Smith (9), that the virus is a hollow protein sphere with the nucleic acid on the inside of the sphere. The nucleic acid-free protein has the same form and diameter as the infectious virus, but actually appears hollow since electron micrographs of air-dried preparations showed flattened spheres with a dimpled surface due to shrinkage. In contrast the air-dried virus has a spherical shape and smooth surface.

The other viruses of this group will probably obey the same structural principles, since the resistance to ribonuclease can best be explained by a centrally located RNA. Crick & Watson (10) assume that the basic requirement for a small virus is the provision of a shell of protein to protect its

<sup>1</sup> The survey of the literature pertaining to this review was completed in September, 1957.

highly specific packet of RNA. This shell consists of a large number of subunits. The number of subunits in a rod-shaped virus is probably unrestricted, but for a spherical virus the number is likely to be a multiple of 12. In spite of the relatively simple structure, the analyses of these viruses have proceeded extremely slowly due to the fact that they can be obtained only in comparatively small quantities.

Chromatography has proved a useful adjunct to the standard methods of virus purification, as shown for example in the work of Shainoff & Lauffer (11) with southern bean mosaic virus, using the strongly basic resin, amberlite.

Many workers have studied the fine structure of the spherical plant viruses by the use of x-rays and electron microscopy. The most important results are the packing of the virus particles in the crystal and the arrangement of the protein subunits in the individual virus particle. Recent studies have been made on the crystalline turnip yellow mosaic virus, by Klug *et al.* (12), by Kaesberg (13), and by Labaw *et al.* (14). The virus particle itself contains a multiple of 12 subunits, probably 60 (15). The bushy stunt virus also seems to be constructed from a series of identical subunits. According to Caspar (16), there are at least 60 such subunits, each having a molecular weight of 125,000. Each crystallographic unit can be subdivided further into a number of chemically identical protein molecules. The crystals of bushy stunt virus can also be demonstrated by direct methods in infected plants [Smith (17)]. The crystalline arrangement and the spacing, 30  $\mu$ , is the same as in the crystals which are obtained *in vitro*.

Steere (18) has studied the tobacco ring spot virus by electron microscopy. The particles appear to be more polyhedral than spherical in shape. The tomato ring spot virus seems to be different from this virus. These particles resemble flattened cylinders with a diameter of 43  $\mu$  and a height of 13.5  $\mu$  [Senseney *et al.* (19); Desjardins *et al.* (20)].

From measurements on a series of electron micrographs of single crystals of the southern bean mosaic virus it was shown by Labaw & Wyckoff (21) that the molecules are in a cubic close packed arrangement. The tetramolecular unit cube has the edge length  $a_0 = 345$  Å corresponding to a dried particle diameter of 245 Å.

Recent chemical investigations on three strains of bushy stunt virus have been made by de Fremery & Knight (22). An RNA content of approximately 16.5 per cent and a similar nucleotide composition was found for all strains. The purine-bound pentose was identified in each case as ribose. The amino acid composition of the three strains was very similar, if not identical. The least frequent amino acid found was cysteine, as in the case of TMV. From the cysteine content a minimum molecular weight of 12,300 can be calculated for the virus protein. An exact test of the uniformity of the peptide chains would be possible by end group analysis. Knight (23) has published some preliminary experiments in which he treated a series of plant viruses

TABLE I  
ENDGROUPS OF PLANT VIRUSES BY TREATMENT  
WITH CARBOXYPEPTIDASE

Virus	C-terminal amino acid
TMV	Threonine
Potato-X	Ala, Asp, Glu, Leu, Thr, Val
Southern bean mosaic	Ala, Leu, Val
Bushy stunt	Val, Leu, Ala
Tobacco ringspot	Ser, Arg
Cucumber viruses	Ala, Leu, Phe, Val

with carboxypeptidase. A clear-cut result was obtained only for TMV where a single terminal amino acid threonine was found. The behavior of the other plant viruses when treated with carboxypeptidase is different in two major respects from TMV (a) several different amino acids were released by treatment with the enzyme and (b) the sum of amino acids released from a given weight was considerably smaller. One can not draw any conclusions as yet concerning the size of the peptide units and their homogeneity in relation to end groups or make any correlations with the crystallographic studies. It is significant that in all cases, the infectivity remains intact after the carboxypeptidase hydrolysis.

#### ROD-LIKE PLANT VIRUSES, EXCEPT TMV

The rod-like plant viruses include the anisodiametric viruses of diameter 10–20  $\mu$  and length 70–1250  $\mu$ . All the viruses in this group which have been chemically analyzed contain RNA. A separate chapter is reserved for TMV, the most studied representative of this group. The nomenclature follows that found in the textbook of Plant Viruses by Smith (24). Electron microscopic observations have not resulted in a very exact determination of the diameters of these viruses. The distribution of lengths of several representatives of this group has been very carefully studied in recent times. These researches show that each virus type possesses a characteristic definite length, designated by Köhler (25) as the normal length. The results of studies obtained from counts on sufficiently large number of particles are summarized in Table II. The homogeneity of the particles is in general dependent on the purification methods, the less extreme methods leading to more homogeneous preparations. The exudate method of Johnson (26) gives exceptionally good preparations. The interpretation of the significance of this normal length has resulted in some controversy. Köhler centers his arguments around the fact that often much larger filaments are found in thin sections than in extracts or exudates. He assumes that these filaments have the tendency to break at certain weak points into segments of the same

TABLE II

NORMAL LENGTH OF RODLIKE PLANT VIRUSES

Virus	Normal length m $\mu$	Authors
Tobacco rattle disease = potato stem mottle	70 and 140 m $\mu$	Paul & Bode (32)
TMV	300	Sigurgeirson & Stanley (33) Williams & Steere (34) Schramm & Wiedemann (35)
Orchid (Cymbium) mosaic	450	Jensen & Gold (47)
Rod-like particles from cactus	500	Amelunxen (49)
Potato-X	510	Bode & Paul (36)
Potato-Aucuba	590	Paul & Bode (37)
Sugar cane mosaic	620	Gold & Martin (48)
Potato-S	650	Wetter & Brandes (29)
Beet mosaic	730	Zimmer & Brandes (40)
	700	Schneider & Mundry (41)
Potato-A	740	Paul & Bode (38)
Potato-Y	750	Bode & Paul (39) Schramm, G. (62)
Bean mosaic	750	Brandes & Quantz (42)
Wisconsin pea streak	750	Skotland (45)
Lettuce mosaic	750	Couch & Gold (46)
Beet yellows	1250	Brandes & Zimmer (43) Burghardt & Brandes (44)

length. Pirie (27) refuses to accept the idea of the occurrence of a normal length, explaining the homogeneity as being due to a selection in the method of preparation. The results with TMV, discussed in some detail below, make it highly probable that primary units of a definite length are formed and that these become heterogeneous later through fragmentation or aggregation. The fragility of the different viruses varies considerably. In some older work [Melchers *et al.* (28)], for example, different particle lengths were found for two strains of TMV. However in a preparation in which fragmentation was kept at a minimum, the particle lengths were identical. The tendency to form aggregates also varies for the different viruses. One always finds, for example, in tobacco rattle virus many particles with a length twice the fundamental length. On the other hand, the tendency of potato-S-virus to aggregate is very slight. The tendency of TMV to aggregate is strongly influenced by the presence of A-protein, which acts as a sort of binding agent. In infected plant cells one can also find noninfectious protein which also tends to form rod-like aggregates. In the case of TMV these aggregates are probably identical with the A-protein. One must be particularly careful in studies with the individual rod-like particles in sap from infected plants. Contamina-

tion with bacteria can result in the presence of fragments of flagella which are similar in shape to rod-like viruses.

The concept of the normal length is further supported by the independence of this length from the host plant, as demonstrated for the sugar beet yellows (43, 44) and beet mosaic viruses (40). Furthermore, serologically related strains of the same virus possess the same length of rods. In every case most authors are in full agreement that the normal lengths, however they may arise, are characteristic for the virus species and can be used to differentiate species as an aid in classification. For example, the potato-S-virus (29), the paracrinkle virus (30), and the carnation latent virus (31) all have the same normal length within the limits of experimental error. Further similarities of various biological characteristics make it highly likely that these three virus types are related to one another. On the other hand the differences in the normal lengths of the beet mosaic and the beet yellows viruses are to be expected from other differences in the properties of these two viruses. An accurately determined length distribution should always be made for newly discovered viruses of this type. This will greatly simplify the problem of relationship to the viruses already classified.

The purification of many rod-like viruses is very difficult since they often tend to aggregate because of their shape. The sugar beet yellows virus can be concentrated without any significant aggregation or fragmentation by centrifugation in a density gradient (50).

#### TOBACCO MOSAIC VIRUS

Tobacco mosaic virus is the best known representative of the group of rod-like viruses, hence its structure and properties will be discussed in some detail.

*Size and form.*—The careful preparation of TMV from tobacco plants yields virus concentrates in which 90 per cent of the particles possess the uniform length of 3000 Å [Williams & Steere (51)]. One can also obtain particles of very uniform length by a single centrifugation of older preparations to remove the A-protein (35). The uniformity of the intracellular virus particles has been confirmed by the electron microscopic studies of Steere (52). Crystals isolated from hair cells can be transected in planes parallel to the rod length. Replicas of the surface can be studied by the electron microscope to show the particles as they are stacked *in situ*. The particles are arranged in palisades and each rod has the length of 3000 Å.

The determination of the molecular weight by physical and chemical methods is difficult since a certain fraction of the particles always form dimers and also the danger of fragmentation cannot be entirely eliminated. The monomer and dimer of TMV have recently been characterized by electric birefringence [O'Konski & Haltner (53); Norman & Field (54)]. From the rotation diffusion constant an average length of  $3416 \pm 50$  Å was determined. Practically the same length,  $3350 \pm 250$  Å, was found by Rowen & Ginoza (55) by measurements of the flow birefringence. Dimerization has



been produced by coupling with methylene blue (56, 57), resulting in the uptake of 3000 protons per monomer.

The detailed internal structure of TMV has been well established by a series of x-ray crystallographic studies as well as by chemical methods. The protein can be separated from the virus by various methods, depending on whether one wishes to preserve the protein or the RNA in the native state. It does not seem possible to preserve both components in the native state by one separation technique. Electron microscopic observations on partially degraded particles have shown that TMV has a central core of RNA surrounded by protein. This was observed by Schramm and co-workers (58) using a mild alkaline degradation and independently by Hart (60) using a detergent for the degradation. The exact spatial relationship between the RNA and the protein has been determined by x-ray crystallography.

*TMV protein.*—The best method of obtaining the TMV protein in a native state is alkaline degradation at pH 10.3 (58). Another method has been recently developed by Fraenkel-Conrat using 67 per cent acetic acid (59). The smallest protein unit found by this procedure is an ultracentrifuge component with  $s_{20} = 4.6$  S, which has a molecular weight of 90,000 from combined sedimentation-diffusion measurements. This unit has been termed A-protein. Under acidic conditions it is able to aggregate to rods of a form and size similar to TMV (61). There are many intermediate stages in this aggregation and each stage can be studied by electron microscopy and ultracentrifugation. Between pH 9 and 7 one finds a protein component with a sedimentation constant of 30 to 40 S, and this component appears in the electron microscope as discs which have a centrally located hole. A further decrease in pH results in the aggregation of these discs to rod-like particles. The length of these rods depends on the pH. The suitable choice of pH leads to the formation of noninfectious rods of the same size and form as the original virus. The A-protein is electrophoretically homogeneous and has a mobility at pH 10.3 of  $-5 \text{ cm.}^2/\text{v. sec.}$  This quantity increases with increase in the degree of aggregation and at neutral pH has the same value as the original virus (309). The alkaline degradation studies of Schramm and co-workers have been extended by Harrington & Schachman (63). Both studies agree on the following points: (a) Approximately 30 per cent of the protein could not be separated from the nucleic acid under the given experimental conditions. This fraction is designated as the stable fraction. (b) Ultracentrifugation of the system during degradation reveals a number of sharp boundaries. Harrington & Schachman have presented a very reasonable explanation for the appearance of these boundaries. They assume that the A-protein is unwound from one end of the TMV rod and after two-thirds of the protein is removed the velocity of the unraveling decreases abruptly to a much smaller value. This stable segment represents the component having a  $s_{20}$  of 133 S. The faster components, especially that of  $s_{20}$  equal to 170 S, arise by aggregation of the stable fragments having  $s_{20} = 133$  S. Certain problems still remain unsettled. For example, according to the hypothesis of Harrington & Schachman the stable segment should have a length of about 1000 A,



whereas electron micrographs show that this segment has a length of 400 to 500 Å (58, 64, 65).

The A-protein is not the smallest protein subunit of TMV. Newmark & Myers (66) have degraded TMV with 0.02 *M* ethanolamine buffer at pH 10.5 and obtained native protein units of molecular weight less than 100,000. Detergents have been employed by Schachman & Hersh (67) to obtain protein units of molecular weight between 10,000 and 20,000.

The size of the peptide subunits can be determined accurately by end-group analysis. Using carboxypeptidase Harris & Knight (68, 69) showed that 1 mole of threonine is released per 17,300 gm. of virus, and Schramm & Braunitzer (70) found practically the same value using another strain of TMV. The enzymatic method of end-group analysis is open to criticism and therefore some workers have used another procedure to determine the C-terminal end group, especially the hydrazine method of Akabori. Again the only end group found was threonine, 1 mole per 15,000 gm. virus (71 to 75). Relatively short exposures to hydrazine (3 to 4 hr. at 100°C.) lead to the isolation of a dipeptide Ala-Thr and a tripeptide Pro-Ala-Thr in the form of their DNP-derivatives. After the threonine is hydrolyzed from the peptide chain by carboxypeptidase the only C-terminal end group found is alanine, also one mole per 16,000 gm. virus. The basic dye O-safranin can be bound to the intact virus, 1 mole per 14,700 gm. virus [Ginoza & Atkinson (76)]. Removal of the C-terminal threonine by carboxypeptidase doubles the O-safranin binding capacity of the virus, one molecule being bound at the original site and the second molecule at the site made available by removal of threonine. Thus each protein subunit possesses a moderately strong acid group which is not identical with the C-terminal threonine.

Studies on the N-terminal sequence of the peptide subunit have been more difficult and complicated since no terminal amino group is detectable in the native virus (77, 78). After treatment of the virus with trichloroacetic acid (pH 1.8, 30 min. at 85°C.) proline can be identified as the N-terminal amino acid, using two different methods, both of which yield proline values equivalent to those of the C-terminal end group. Braunitzer (79) has studied the N-terminal end group in more detail in order to explain why it cannot be detected in the native TMV. The amides of the gamma-carboxylic group of glutamic acid and the beta-carboxylic group of aspartic acid are more labile than the peptide bond, as is well known. Braunitzer argued that the masking of the N-terminal group was caused by such a beta- or gamma-peptide linkage of a side chain and that this was split by the trichloroacetic acid treatment. This type of peptide linkage can also be easily split by hydroxylamine with the formation of the corresponding hydroxamic acid. Thus treatment of TMV with 3*N*  $\text{NH}_2\text{OH}$  at pH 7 (24 hr. at 60°C.) resulted in the exposure of 60 to 80 per cent of the theoretical amount of N-terminal proline, without a significant degree of splitting of other peptide bonds. Further experiments, still in progress, suggest that the masking is due to the beta-carboxylic group of aspartic acid.

The most probable value for the peptide subunit is 16,500, which is in

good agreement with the amino acid analysis of Knight (80, 81). On the assumption that each chain contains one molecule of cysteine, the molecular weight is 16,300 with approximately 150 amino acids in the chain.

The amino acid sequence of the peptide chain is now being studied. Chymotrypsin hydrolysis of the peptide followed by paper chromatography of the DNP-peptides enabled Niu and Fraenkel-Conrat (82) to determine the C-terminal sequence of 4 different strains of TMV. The sequence Thr-Ser-Gly-Pro-Ala-Thr was found in normal TMV and strains M and YA, but strain HR had the sequence Thr-(Thr, Ala)-Pro-Ala-Thr. After treatment with trichloroacetic acid, Braunitzer (72) found the N-terminal sequence Pro-Ileu-Glu, whereas Anderer (83) found the above and a second sequence: Pro-Leu-Val. It is not yet clear whether this discrepancy is due to inhomogeneity of the strain or to the existence of different peptide units in the virus protein. New data concerning the amino acid composition of different strains of TMV and their serological relationship are presented by Aach (310); the electrophoretic mobilities of these strains and their A-proteins were investigated by Kramer (309).

*Relation of A-protein to abnormal proteins in infected plants.*—Takahashi has isolated a protein from TMV-infected tobacco plants which is noninfectious, but reacts with anti-TMV serum. This was designated the X-protein. Subsequently more components with different electrophoretic mobilities were found (84 to 87). According to Schramm & Zillig (61), the X-proteins and the A-protein or its polymerization products are very similar, if not identical. The A-protein, obtained by viral degradation, is serologically inhomogeneous, but the bulk of the material is identical with the X-protein [Kleczkowski (88)]. The slight differences are possibly due to different degrees of polymerization (89, 90). Furthermore, the amino acid compositions of the X-protein and the A-protein are very similar. X-protein was isolated from plants infected with the normal strain of TMV as well as with other strains (86). X-proteins from these other strains have different electrophoretic mobilities than that from normal TMV. According to Bawden & Pirie (91) the X-proteins may also contain a small amount of phosphorus. The serological relationship of the A- and X-proteins to the intact virus has been studied by Starlinger (92). Antiserum absorbed by A- or X-protein still precipitated TMV. This serological difference is not due to the viral RNA since the absorbed antiserum is neither precipitated nor blocked by the viral RNA.

Several studies have attempted to answer the question whether the X-protein is a precursor or degradation product of TMV (93, 94).  $N^{15}$ , added as  $NH_4Cl$ , is incorporated uniformly into TMV and into X-protein. However,  $C^{14}$  as  $CO_2$  is incorporated into the X-protein at a much higher rate than into the virus. The specific radioactivity of the virus calculated on the basis of the hypothesis that these abnormal proteins are the immediate precursors of the protein part of the virus is very close to the measured radioactivity (95). The serological differences between A- and X-protein, men-

tioned above, also suggest that the X-protein is a precursor rather than a degradation product of the virus.

**Nucleic acid of TMV.**—There are several well-known methods to isolate the RNA from TMV. Besides the older method of Cohen & Stanley (96) (heating to 100°C. for 1 min.) there is the newer technique of Fraenkel-Conrat and co-workers (97, 98) in which a 1 per cent TMV solution is heated in a 1 per cent sodium dodecyl sulfate (Dupanol) solution (50°C., 5 min.). This is followed by precipitation of the protein with ammonium sulfate and by further purification of the RNA by alcohol precipitation. Amino alcohols of the general formula  $R'R''N-CH_2OH$  can also be used in the isolation of the RNA (66). A very simple procedure is the extraction by phenol of a TMV solution having a low salt concentration [Schuster, Schramm & Zillig (99)], Gierer & Schramm (100). The protein passes into the phenol phase and the protein-free nucleic acid remains in the aqueous phase. When the RNA is extracted with phenol at low temperatures, the protein-free end product still possesses considerable infectivity, about 5 per cent of that of the equivalent weight of virus or 0.3 per cent of the virus infectivity calculated from the weight of the infectious RNA and the equivalent weight of RNA contained in the virus itself. Approximately the same infectivity (1 to 5 per cent) was reported by Fraenkel-Conrat for the RNA prepared with dodecyl sulfate. The RNA prepared by the phenol method contained no detectable protein (less than 0.4 per cent) and repeated extractions with phenol did not decrease the biological activity. Thus the virus protein is not essential for infectivity. Further control experiments have excluded the possibility that small amounts of infectious virus still remained in the RNA. The RNA activity is not affected by TMV antiserum, but the activity is rapidly destroyed by ribonuclease whereas TMV is resistant to the enzyme. In comparison with TMV, the RNA activity is very labile. Frequently a loss in infectivity is observed after 2 hr. at room temperature. The cause of this inactivation is not yet clear.

The RNA is not completely homogeneous in the ultracentrifuge. In addition to the homogeneous component A there are components which migrate slower and are seemingly degraded. Component A has a sedimentation constant of 19 S in a 0.2 per cent solution in 0.02 M phosphate buffer at pH 7. This constant increases with decreasing concentration of nucleic acid. From the  $s_{20}$  values and the intrinsic viscosity, the molecular weight of component A was found to be  $2.1 \times 10^6$ , corresponding to the intact RNA core within the limits of accuracy. The RNA possesses a significantly higher optical rotation and an approximately 20 per cent lower ultraviolet absorption than the sum of its nucleotides [Gierer (101)]. The sign of the birefringence is positive as in the virus indicating that the bases are parallel to the long axis of the molecule [Ginoza & Norman (102)]. These results suggest that the RNA has a highly ordered structure. The shape of the RNA is strongly dependent on the ionic strength of the medium, as is true for other polyelectrolytes. The  $s_{20}$  value decreases sharply in distilled water, whereas

the viscosity rises. As expected from theoretical considerations, this phenomena demonstrates a significant elongation of the molecules in distilled water. By differential centrifugation it was shown that the sedimentation constant of the infectious component is the same as that of component A within  $\pm 25$  per cent. Degradation of the high molecular weight RNA with ribonuclease causes a decrease in the viscosity and a simultaneous decrease in the infectivity. This shows that the biological activity is related to the high molecular weight component. Quantitative studies of the degradation with ribonuclease lead to the conclusion that a few or perhaps a single break are sufficient to destroy the activity of component A. Smaller fractions obtained by the action of ribonuclease are inactive. From sedimentation analyses carried out on RNA in distilled water, Fraenkel-Conrat *et al.* (98) have concluded that low molecular weight RNA is also infectious. However no viscosity or diffusion measurements were made on the RNA in distilled water and hence the authors cannot determine the molecular weight under their conditions.

The radiosensitive volume of TMV and infectious RNA, determined by x-ray inactivation, is in good agreement with the idea that only the RNA is responsible for the virus multiplication (102, 103). From the results it can be concluded that the radiosensitive molecular weight of the infectious nucleic acid of TMV is about  $2.7 \times 10^6$  and that the total RNA component of the virus behaves as a single infectious unit. Inactivation of TMV with x-rays results in a decrease in the particle weight of the RNA component (104). Thus in agreement with the degradation studies of Gierer (101) the smaller units of RNA are no longer infectious.

The ultraviolet sensitivity of RNA from different TMV strains has been measured by Siegel *et al.* (105) and by McLaren *et al.* (106). Isolated RNA from strain U-I, corresponding to normal TMV, is six times more sensitive to ultraviolet light than the intact virus. However, there is no difference in the ultraviolet sensitivity of the RNA and the virus for strain U-II. The inactivation is by a single-hit process. A general discussion of the effects of non-ionizing radiation on viruses has recently appeared (107).

Some other physical studies on the RNA from TMV have been made, but on noninfectious material, hence on material which is probably partially degraded. Light-scattering measurements on RNA prepared by a modification of the method of Cohen & Stanley gave a molecular weight of  $1.7 \times 10^6$  [Hopkins & Sinsheimer (108)]. Further studies on partially degraded RNA by Schuster *et al.* (99) have shown that the sedimentation constant and viscosity decrease with decreasing RNA concentration due to a dissociation of aggregates. Urea favored dissociation and high salt concentration favored association, precipitation occurring in approximately 1 M NaCl. Electron microscope studies were also made on different RNA preparations, but no exact determination of the diameter and length could be obtained since the fibers aggregated strongly.

The chemical composition of the RNA has been studied in many labo-

ratories. Reddi & Knight (109) have attempted to determine the end groups, but this has been unsuccessful although the method used was so sensitive that one end group per 500 nucleotides could have been detected. Older findings in disagreement with Reddi & Knight were made by Matthews *et al.* (110), but have now been retracted by the authors themselves (111). The virus RNA is clearly distinguished from the RNA of normal tobacco plants by its content of uracil and guanine (112). No significant differences were found in the RNA content or base composition of the normal and Holmes masked strains (113). Phosphoprotein, phospholipides, and DNA in amounts of greater than 1 per cent were not detected. Small amounts of metals have been detected, probably bound (114) to the RNA. Degradation of RNA with ribonuclease yielded a purine-rich core consisting of about six nucleotides and this core seems to be identical for all strains as yet studied [Reddi & Knight (115); Reddi (311)]. In contrast to these findings, Commoner & Basler (116) found highly variable values for the RNA content and base composition in different preparations from the same strain.

If infected tobacco leaves are incubated in a medium containing thiouracil, then this is incorporated into the TMV. This was previously reported by Jeener & Rosseels (117) and recently confirmed and further studied by Matthews (118). The amount of incorporation corresponded to about 3.5 per cent of the uracil, which is about the same proportion of the normal base as that found for 8-azaguanine in TMV [Matthews (119)]. Thiouracil replaces the uracil in several positions, but probably mostly at the end of the polynucleotide chain (120). The thiouracil treatment blocks the virus multiplication to a certain extent, although virus containing thiouracil give the same number of local lesions per gram of virus on *Nicotiana glutinosa* as the normal virus [Jeener (121)]. Since only 4 to 18 per cent of the uracil is replaced by thiouracil a noticeable effect is hardly expected. The multiplication of the thiouracil virus is considerably delayed in comparison with that of normal virus. From these observations Jeener reaches a conclusion which is difficult to understand. Namely that the infective material in a single virus particle is reduced by the incorporation of thiouracil. It would be much simpler to assume that the presence of the thiouracil virus increases the lag phase without influencing the number of infective centers. The effects of other purine and pyrimidine analogues were discussed by Matthews & Smith (122).

The inactivation of infective centers with ultraviolet light has yielded some information on the lag phase of different strains of TMV grown in *Nicotiana glutinosa*. The change in sensitivity of the infective centers to ultraviolet light was followed as a function of time after inoculation. There is a period after inoculation during which no change in ultraviolet sensitivity occurs. This lag period is about 2.5 hr. for the U-II strain and somewhat over 5 hours for the U-I strain. In contrast, when nucleic acid is the infecting agent, two marked differences are apparent: (a) the lag period is greatly reduced or missing entirely, and (b) identical behavior is exhibited by the

nucleic acid infective centers of both strains [Siegel, Ginoza & Wildman (123)]. This indicates that the initial events of TMV infection are concerned with the release of the nucleic acid from the protein moiety of the virus particle. Physical and chemical studies of the extracellular material revealed a greater affinity between nucleic acid and protein in the U-I strain, this is reflected in the increased lag phase of this strain. The experiments of Hamers-Casterman & Jeener (124) on the initial ribonuclease sensitive phase in the multiplication of TMV are further proof that the RNA is separated from the protein before multiplication begins.

There has as yet been no clear cut location of the intracellular centers of virus multiplication. Using ultraviolet-microspectroscopy, Zech & Vogt-Köhne (125) showed that the ratio (RNA+DNA)/protein increases first in the nucleus and then in the cytoplasm. However it is not possible to draw any conclusions concerning the production of virus specific RNA from spectroscopy alone. The number of TMV particles in one infected cell is extremely high,  $6 \times 10^7$  particles/250,000  $\mu^3$  (126) have been calculated from particle counts in ultrathin cell sections. This high virus concentration makes the prospects for further studies on the virus multiplication very favorable. Further electron microscope studies on infected cells have been made by Brandes (127).

Chemical studies on the RNA content of infected plants have been made by Basler & Commoner (128). Worth noting is the work of Wüstringer *et al.* (129) showing that  $C^{14}O_2$  is incorporated into the RNA earlier than into the protein. After the destruction of the cells the incorporation stops at once (130).

**Reconstitution.**—The A-protein of TMV is easily bound to high molecular weight RNA from various sources. It can even form rods with synthetic polyuridylic acid prepared using Ochoa's polynucleotide phosphorylase [Hart & Smith (131)]. Hence the combination of A-protein with the RNA is not a specific reaction and the question as to whether recombinants can be considered as reconstituted virus remains open. Recombinants can be distinguished from normal virus by their ultraviolet sensitivity (105).

According to Fraenkel-Conrat & Williams (97), noninfectious RNA prepared with sodium dodecyl sulfate can be reconstituted with A-protein to form infectious virus. Later, when the infectivity of the RNA alone had been recognized, this reconstitution experiment was interpreted as a stabilization of the RNA (98). After addition of A-protein the activity of the RNA became resistant to ribonuclease. Since the free RNA is some 300 times less active than TMV, complete reconstitution should result in an increase in the total activity by the factor 300. However, due to the instability of the RNA an exact determination of the reconstitution factor has not yet been possible. Reconstitution of the RNA with A-protein of the same or of another strain has been reported by Fraenkel-Conrat *et al.* (132, 133, 134), and by Lippincott & Commoner (135).

A considerable increase in the activity may be produced with particles



which have lost only a portion of their protein coat. Particles partially degraded in hot detergent could be reactivated by a factor 10 [Hart (136)]. But Commoner *et al.* (137) found a thousandfold increase in infectivity when A-protein was combined with RNA. This high factor can be easily explained since the control experiments were performed only after the RNA activity had practically disappeared. Further experiments from Wang & Commoner (138) report the formation of infective TMV from DNA of noninfected tobacco plants mixed with A-protein, an observation which still requires confirmation, since it is well known that a large excess of A-protein can inhibit TMV and that after removal of this excess of A-protein a small residual amount of infectious TMV can be demonstrated. Bawden & Pirie (139) made the following critical comments on the results of the reconstitution experiments: The possibility that infective TMV can be reassembled *in vitro* from previously noninfective components cannot be excluded, but all the results that could be interpreted as suggesting this are also interpretable in other ways, either by the removal of inhibitors of infections or by the stabilization of infective fragments which otherwise would have become inactive before testing.

**X-ray structure analysis.**—The chemical studies on the structure of TMV have been refined by the x-ray crystallographic analysis of TMV and the RNA-free A-protein. This showed TMV to be a hollow cylinder of external diameter 170 Å and internal diameter 40 Å [Franklin (140); Caspar (141)]. The cylinder is constructed of subunits having a molecular weight of 16,000 and arranged in a helix of pitch 23 Å. There are  $3n+1$  subunits per 3 turns (142), with the latest value of  $n$  being 16 (143, 144). The nucleic acid is embedded in the hollow cylinder, the electron dense P atoms are located some 40 Å from the center. There is a groove some 30 Å deep on the surface of the rods and this groove follows the protein helix. In the close packed state the TMV rods become dovetailed with each other so that the distance between centers is some 150 Å. The particles fit together, ridge to hollow, like machine screws of the same pitch (145). From x-ray small angle scattering measurements, TMV in solution has a circular cross section of 167 Å diameter [Kratky *et al.* (146)] which, within the limits of error, is in good agreement with the values obtained from oriented preparations. Small angle scattering measurements on cucumber virus 4 by Kilkson (147) also gave a cross sectional diameter of 164 Å. The x-ray diffraction patterns of this virus and several TMV strains are very similar (148, 149). The aggregated A- and X-protein also have diffraction patterns very much like that of TMV with the same external diameter and pitch, but the characteristic electron dense shell at 40 Å representing the nucleic acid P is absent (150, 151, 152). The formation of the nucleic acid free rods is probably as follows: 6 peptide subunits aggregate to form the A-protein units and 8 such units aggregate to form a protein disc 69 Å thick; these discs then polymerize to form the complete rods (143). A central hole could be demonstrated in the discs. The existence of the predicted hollow cylinder along the axis of the TMV rod



has been elegantly confirmed [Huxley (153)] by allowing some electron-opaque material to enter the cylinder and dry, leaving a coating of dense metal along its inner wall. The above-mentioned groove on the surface of the TMV has not yet been observed in the electron microscope. A repeating surface structure of 46 Å period has been observed running along the long axis [Matthews *et al.* (154)]. Also shadowing at a very low angle has revealed a period of 136 Å (155). The connection between these observations and the x-ray structure surface groove has not yet been explained.

*Chemical reactions of TMV.*—Since TMV is the most convenient virus to purify in large quantities it has been used a great deal as a model for general reactions. Of special importance is the reaction with formaldehyde since this reagent is used so often for vaccine production. Although the RNA is protected from the external medium by a protein coat it is nevertheless attacked by formaldehyde. The inactivation rate of the virus is not changed when all sulfhydryl and reactive amino groups are previously blocked by oxidation and acetylation respectively (156, 157). When the RNA was separated from the formaldehyde treated TMV it was found to contain about 0.8 per cent  $\text{CH}_2\text{O}$  compared to 0.05 per cent in the protein moiety (156). This appears to represent definite proof that the nucleic acid of the virus reacts with formaldehyde. According to Staehelin (158) the infectivity of the RNA isolated from TMV was found to be very sensitive to  $\text{CH}_2\text{O}$  treatment. Experiments with  $\text{C}^{14}\text{H}_2\text{O}$  show that two different reactions take place. The greater part of the formaldehyde is bound in a reversible form and lost on dialyses, while a smaller part is more firmly bound and cannot be removed by dialysis. It is well known that TMV is not inactivated when the sulfhydryl groups are gently oxidized with  $\text{I}_2$ . In this case Fraenkel-Conrat (159) showed that the reaction  $\text{—SH} + \text{I}_2 \rightarrow \text{—SI} + \text{HI}$  occurs, a reaction not previously observed with other proteins. The sulphenyl iodide is stable in native TMV, protected against hydrolysis. A hydrolysis to sulfoxide or a transformation to disulfide linkage occurs only after denaturation. The reaction of iodine with the sulfhydryl groups does not influence the serological specificity of TMV. An effect on the antigen structure is observed only after further treatment with  $\text{I}_2$  in which tyrosine becomes iodine substituted (160). The kinetics of the antibody inactivation of TMV have been studied in great detail by Rappaport *et al.* (161, 162).

#### INSECT VIRUSES

There are some well-known viruses which multiply in plants as well as in insects (leafhoppers) and which are usually designated as plant-insect viruses. Typical examples are the aster yellows virus and the curly top virus. Very little is known concerning the biochemistry of these viruses since they are extremely difficult to purify [Maramorosch (163)].

The morphology and development of the typical insect viruses have been recently reviewed by Smith (164). Insect viruses can be classified in three types, distinguished by their symptomatology: (a) the polyhedral virus diseases, which are subdivided into nuclear polyhedroses and cytoplasmic

polyhedroses; (b) the granuloses or capsular virus diseases; and (c) viruses without intracellular inclusions.

This reviewer will only discuss several of the recent chemical studies with these viruses. The most characteristic property of the viruses of groups *a* and *b* is that the infectious particles are surrounded by a large mass of noninfectious protein, the origin and meaning of which is not clear. In group *a* the protein forms polyhedral crystals which enclose a large number of virus particles. The virus is sometimes arranged in closely packed bundles. In group *b* one sees capsules which contain only 1 or at most 2 virus rods. Wellington (165) has made comparative amino acid analyses on a series of polyhedra and capsules and on the corresponding virus particles. The composition of the noninfectious protein is similar for the different types but is clearly different from the protein of the infectious particles.

The paracrystalline lattice of the polyhedral bodies of *Prothetria dispar* (gipsy moth) and *Bombyx mori* (silkworm) was examined in ultrathin section in the electron microscope [Morgan *et al.* (166)]. The polyhedral proteins are prolate ellipsoids with a molecular weight of about 276,000 and 378,000 respectively and an axial length of 180 Å for *B. mori*, closely packed in an orthorhombic unit cell. Scattered within the matrix are bundles of virus particles. The gipsy moth virus occurs in bundles of one to eight rods enclosed by a limiting membrane. The particles of silkworm virus, although generally occurring singly, also possess a limiting membrane. If the polyhedra are dissolved in weak alkali one can recover virus particles which are enclosed in a membrane. According to the studies of Bird (167) this membrane has a certain function in the developmental cycle. Smith & Xeros (168) agree that the virus rod is enclosed by a membrane, but they believe that the membrane is acquired after the rod has been formed.

With an increase in the concentration of alkali the membranes burst and the released virus particles are split into subunits. In *Aporia crataegi* noninfectious subunits with a diameter of 50  $\mu$  and a central hole, thus very similar to TMV, have been observed [Krieg (169)]. These viruses are probably built up from protein discs, much like the A-protein aggregation, but which are then held together by a membrane.

It is interesting from the chemical viewpoint that the polyhedral viruses always contain DNA as nucleic acid (170). Enzymes, especially deoxyribonuclease, have not yet been found associated with the virus (171). The silkworm virus seems to contain small quantities of Fe and Mg (172). The cytoplasmic type of viruses, in contrast to the nuclear, are mostly spherical and contain RNA [Krieg (173)]. Virus particles obtained from the cytoplasmic polyhedra of *Dasychira pudibunda* contain 6.7 per cent RNA. Only RNA has been detected in a histochemical study on the cytoplasmic polyhedroses of *Thaumatopea pityocampa* [Xeros (174)] and in *Sphinx populi* chemical studies lead to an RNA content of 0.9 per cent.

A cytoplasmic virus in the group of viruses without intracellular inclusions has been isolated from larvae of *Tipula paludosa* (crane fly) [Williams & Smith (175); Smith & Xeros (176); Xeros (177)], and shown to contain

DNA. This virus can be used for further physical and chemical studies since it can be easily obtained in larger quantities (5 to 10 mg. per larva) in a fairly pure form. The individual virus particles are polyhedral in shape with a diameter of approximately 100  $\mu$ . By concentration of aqueous solutions the virus can be crystallized into a network in which the center to center particle distance is 130  $\mu$ .

#### ANIMAL VIRUSES LESS THAN 50 $\mu$ IN DIAMETER

The small animal viruses are here divided into three groups for purposes of discussion: (a) poliomyelitis, EMC, Cocksackie, and ECHO viruses; (b) arthropod-borne encephalitis viruses; and (c) other viruses. The first group comprises relatively uniform particles of diameter 28  $\mu$ . The particles of the second group are also spherical, but vary in size.

All viruses of groups *a* and *b* contain RNA and no DNA. Enzymatic activity has not been found associated with the virus particles. Most of the newer studies on these viruses have employed the plaque assay of Dulbecco and Vogt for quantitative determinations of numbers of infectious particles. The most important advance in the biochemistry of these viruses is the crystallization of polio (178) and Cocksackie (179) viruses, which also shows that they possess a morphology comparable with that of the spherical plant viruses. An infectious RNA fraction has been prepared from several representatives of this group: Eastern Equine Encephalitis [Wecker & Schäfer (180)], Western Equine Encephalitis [Wecker & Franklin (181)], Mengo Encephalitis virus [Colter *et al.* (182)], Poliomyelitis and West Nile virus [Colter *et al.* (312)], all using the phenol method employed with TMV. This nucleic acid fraction seems to be protein free and produces the characteristic symptoms of the virus disease when inoculated into test animals. Thus one may conclude with some certainty that the RNA is the carrier of the genetic information as in TMV.

*Poliomyelitis group.*—The purification, identification, and characterization of poliomyelitis virus types 1, 2 and 3 are the result of investigations carried out in the last years by Schwerdt & Schaffer (183, 184). The source of virus for these studies was infectious fluid from tissue cultures of monkey kidney cells. Assays for following the course of infection were carried out by the plaque technique on monolayer cell cultures. The essential steps for purification were as follows: (a) Precipitation of the virus from tissue culture fluid with 15 per cent methanol at pH 4 and elution of the precipitate with one-fiftieth volume of molar NaCl solution at pH 9; (b) two extractions with *n*-butanol; (c) reprecipitation and elution of virus as in step *a* but without methanol; (d) one cycle of high- and low-speed ultracentrifugation; (e) treatment with crystalline ribonuclease and deoxyribonuclease; (f) a final cycle of high- and low-speed ultracentrifugation. Further purification of the virus concentrates was effected either by moving boundary electrophoresis or by ultracentrifugal sedimentation in a sucrose density gradient. The conditions for crystallization were sedimentation of the virus by ultracentrifuge

and storage of the gel-like pellet for several days. The virus could be recrystallized without loss of specific activity. The purest preparations of the three poliomyelitis strains (Mahoney, MEF-1 and Saukett) were analyzed ultracentrifugally, giving homogeneous bands with  $s_{20} = 160$  S. The partial specific volume of the particles was determined by ultracentrifugation in mixtures of  $D_2O$  and  $H_2O$  in the presence of  $0.14$  M NaCl. The density found,  $1.56$  to  $1.61$ , is very high when compared with the value of  $\sim 1.33$  usually assumed for protein. From the  $s_{20}$  and  $V_0$  values the diameter of the water free virus was calculated to be  $24$   $m\mu$  in contrast to the value  $27$   $m\mu$  found for the hydrated virus by electron microscopy. Further calculations showed the molecular weight to be  $6.8 \times 10^6$  and the particle weight in grams to be  $1.13 \times 10^{-17}$ . Hydration results in the binding of  $0.3$  gm. water/1 gm. dry weight. No physical differences could be demonstrated between the individual strains. In freshly purified preparations approximately 30 to 35 physical particles were found equivalent to one plaque (185, 186). Quantitative orcinol tests revealed an RNA content of 22 to 30 per cent, and no DNA was detectable. No thymine could be detected by hydrolysis and subsequent chromatography. Only slight differences were found in the molar ratios of the other bases of Mahoney and MEF-1 strains. Carbohydrates were not detectable. Furthermore the stability of infectivity in the presence of organic solvents and the apparent high dry weight of the virus particles suggest the absence or low content of lipides.

When partially purified virus suspensions are fractionated in a density gradient, slower sedimentating components are found which contain a measurable amount of antigenicity, but no infectivity. These antigens are type specific and give no precipitation with heterotypic or antinormal cell sera. One of these noninfectious antigens exhibited a typical nucleoprotein spectrum, while the spectrum of the other fraction indicated the presence of little or no nucleic acid. Electron micrographs of this fraction revealed flattened particles of low electron opacity.

According to Polson & Selzer (187), there are two types of infectious particles for poliomyelitis strain MEF-1, with diameters of  $24$  and of  $30$   $m\mu$ . After separation, the smaller particles can be further passaged in suckling mice without the reappearance of the larger particles. The size determinations were carried out in a preparative ultracentrifuge using haemocyanin as a comparative standard. Since this method is not very reliable one must await further confirmation of this finding. Polson *et al.* (188) have further demonstrated a soluble antigen which may be separated from the infectious particles by ultracentrifugation but not by electrophoresis.

For the crystallization of Cocksackie virus the A-10 strain, capable of multiplying in suckling mice, was used by Mattern & DuBuy (179). One volume of carcasses was homogenized in two volumes of 8.6 per cent sucrose and cleared of subcellular particulates. The virus was further purified by precipitation with ammonium sulfate and alcohol and by ultracentrifugation. Finally the pellet was stored at  $4^\circ C$ . and crystals formed in a manner

similar to poliomyelitis virus. Upon recrystallization, crystals 0.1 mm. in diameter could be obtained. The crystals are extremely unstable and have been maintained only in suspension. They resemble the unstable crystals seen in plants infected with TMV. The diameter of the individual particles is about 28  $\mu$ . Chemical analyses have not yet been carried out. According to these studies the Cocksackie and poliomyelitis viruses are very similar in their physico-chemical properties. The fact that all of the enteric viruses including the poliomyelitis, ECHO, and Cocksackie viruses have many properties in common suggests that they might have a common evolution [Melnick (189)]. The biological properties of these viruses, especially the growth cycle will not be discussed in this review. A good summary of this topic can be found in the reprints of the 4th International Poliomyelitis Congress (189). This report also contains detailed medical reports of the results of extensive studies on the poliomyelitis vaccine developed by Salk.

*Arthropod-borne encephalitis viruses.*—The equine encephalitis viruses are the best studied of the group of encephalitis viruses. They have a diameter of 40 to 50  $\mu$ , contain a great deal of lipide, and only RNA as nucleic acid. A study of the basic aspects of neutralization of western equine encephalitis and poliomyelitis was carried out by Dulbecco *et al.* (190). The method developed by Gierer & Schramm (100) was used for the isolation of infectious nucleic acid from cells infected with eastern equine encephalitis or mengo encephalitis. Because of the sensitivity of the nucleic acid to enzyme degradation, special precautions must be taken to prevent cellular ribonuclease from acting during the isolation procedure. Control experiments carried out in a similar fashion to those with TMV showed that the infectivity cannot be due to residual amounts of infectious virus. The RNA preparations are some 1000 times less active than the starting infectious material, probably because the test cells are less receptive or sensitive to the infectious RNA than to the intact virus. The question remains open as to whether this infectious nucleic acid is isolated from the virus itself or from a precursor. Data are presented which indicate that, in the case of RNA preparations isolated from mengo encephalitis virus infected Ehrlich tumor cells, the infectious component arises from intact virus particles (312).

*Other viruses.*—The papilloma virus, with a diameter of about 40  $\mu$ , is clearly distinguished from the other viruses of this group in that it contains only DNA in a concentration of 8.7 per cent. For several other small viruses Polson & Selzer (188) found infective particles of various sizes: African horse sickness—24, 30 and 50  $\mu$ , Rift valley fever neurotropic strain 30 and 50  $\mu$  and pantropic strain 50  $\mu$ , Yellow fever, Semliki forest, and West Nile 50  $\mu$ .

#### ADENO-VIRUSES

The adeno-viruses seem to represent a special class. Of great interest are the electron microscope studies of Morgan *et al.* (191). Cells infected with adeno-viruses exhibit intranuclear crystals composed of viral particles that are 60  $\mu$  in diameter and packed in a cubic body-centered lattice. The

determination of the crystal packing structure has been discussed in some detail by Low & Pinnock (192). Comparative electron microscopical and histochemical studies have shown that the crystals are Feulgen positive, suggesting that the virus contains DNA [Block *et al.* (193)]. Although the particles in the crystals are 60  $\mu$  in diameter, isolated shadowed crystals are 90  $\mu$  in diameter, probably due to flattening of the particles upon drying (194). Besides the DNA-containing virus particles, in some cases a crystalline protein occurs in infected cells which is free from DNA (313, 314).

#### MYXO-VIRUSES

The myxo-viruses proposed by Andrewes (195), comprise viruses which possess enzymatic activity. They may be divided into two groups according to morphology (a) influenza and fowl plague viruses and (b) Newcastle disease and mumps viruses.

*Influenza and fowl plague viruses.*—The viruses of influenza and fowl plague are very similar in form and structure [Burnet (196)]. Both are spherical with a diameter of 70 to 80  $\mu$ . Recent sedimentation studies confirm older work [Pye *et al.* (197)]. The purification of these viruses is difficult. Recently chromatographic procedures have been employed for this purpose. By employing strongly basic ion exchange media Matheka and Armbruster (198) found that certain strains of influenza could be separated into several infectious components. Adsorption on aluminium phosphate can also be used for differentiation and purification (199). Both viruses contain RNA as the only nucleic acid, influenza having about 1 per cent (200, 201, 315) and fowl plague about 3 per cent (202). Since these viruses are so difficult to purify, these differences should not be considered confirmed. Besides ribose, the sugars galactose, mannose, fucose and glucosamine have been found in influenza. With the exception of ribose, the same carbohydrates are found in a normal component of allantoic fluid, which can be separated from the virus only with great difficulty [Frommhagen & Knight (203); Ada & Gottschalk (204)]. Schäfer (205) has been able to show that there are also certain serological relationships between influenza and fowl plague.

The structure of the particles has been revealed largely through the separation of the virus into several components by ether degradation, first made by Hoyle (206) on influenza and further refined by Schäfer and co-workers (202, 207) with fowl plague. Lipide is found in the ether phase, and two components, no longer infectious, are found in the water phase. The first component, the "gebundenes Antigen" (g-antigen) is identical with the soluble antigen (s-antigen) which is a virus specific product always found in infected cells. The g-antigen particles have a diameter of 10 to 15  $\mu$  and contain some 10 to 15 per cent RNA plus protein. The second component, the hemagglutinin, bears the enzymatic activity against mucins. The fowl plague hemagglutinin has a diameter of 30  $\mu$ , whereas the influenza hemagglutinin has a diameter of only 12  $\mu$ . The hemagglutinin contains carbohydrate and protein. Serological studies have shown that the nucleic acid containing g-antigens of both influenza [Lief & Henle (208)] and fowl plague



(207) are located in the interior of the elementary particles. Antisera to g-antigen do not react with intact virus particles and vice versa; antisera to intact particles do not react with g-antigen. The g-antigens of influenza and fowl plague cross react serologically although the intact virus particles do not.

Infected cells and tissues contain incomplete forms as well as s-antigen and the elementary particles (209). These non-infectious hemagglutinating particles have also been designated NIHA-forms (210). The incomplete forms of fowl plague have been isolated and studied in the electron microscope. They are low contrast particles, much like empty sacks or balloons, of highly variable diameters between 50 to 550 m $\mu$  (211). The incomplete forms of influenza are distinguished by a high lipide content (54 per cent) (212) and a low RNA content (201).

Filamentous particles are also found in infected cells. They can attain lengths greater than 1  $\mu$  and have a thickness of about 80 m $\mu$ . They have been studied in detail by Valentine & Isaacs (213). According to the ultra-histological studies of Schäfer & Hotz (214) the filamentous forms of fowl plague are hemagglutinating. It is not yet certain that they are infectious (215).

Both the filamentous forms and the incomplete particles appear at the cell membrane shortly before the appearance of new infectious particles. One has the impression that these forms are protusions of the cell membrane. Most investigators are of the opinion that the spherical infectious particles are formed in these protusions, but Morgan *et al.* (216) claim that the elementary body can be formed independently of the protusions on the cell surface. Thin sections of influenza virus reveal an electron-dense central region surrounded by a region of lower electron-density, and this enclosed by a membrane. The electron dense center probably corresponds to the g-antigen containing the nucleic acid, the less dense area to the hemagglutinin, and the membrane to the lipide.

The growth cycle of these viruses is complicated, as would be expected from the complicated structure. Employing the Coons' fluorescent antibody method, Breitenfeld & Schäfer (217) have shown that the g-antigen appears first in the cell nuclei. Hemagglutinin synthesis commences in a certain region of the cytoplasm, near the nucleus, and then spreads over the cytoplasmic reticulum. The two viral components probably unite near the cell membrane, where an especially strong fluorescence is observed toward the end of the latent period. These experiments have been extended by Franklin (218) using macrophages and multinucleate giant cells as host. Location of the g-antigen only in the nuclei was nicely demonstrated in the multinucleate cells.

Experiments with radioactive isotopes, especially P<sup>32</sup>, should be valuable in elucidating further details of the growth cycle. Such studies have been made on influenza virus by Liu *et al.* (219) and on fowl plague by Wecker (220). The specific activity of fowl plague depends on how early the isotope is added to the tissue culture before the start of infection. Variations in this



time result in variations in the  $P^{32}$  content of the phospholipide fraction but the  $P^{32}$  of the RNA fraction remains constant. This suggests that  $P^{32}$  is incorporated into viral RNA rapidly and by a fairly direct pathway whereas the incorporation into lipides is through cellular components which later enter the virus particle. The infection of cells with  $P^{32}$  labeled virus shows that immediately after infection there is a disintegration of the intact virus leading to a release of the nucleic acid containing g-antigen [Wecker & Schäfer (221)]. This is in agreement with the observation that ribonuclease can block the synthesis of influenza virus although the complete virus is resistant to the enzyme (222). When one cell is infected with two different strains of influenza virus a recombination occurs as in bacteriophages (223, 224).

The first stage of the infectious process in which the influenza virus adsorbs to the surface of the host cell is that most easily analyzed biochemically. The adsorption of the virus on erythrocytes is usually used as a model of this system. This process is intimately associated with the neuraminic acid content of the cellular receptors. After adsorption, neuraminic acid is split from the receptor site by the enzyme of the influenza virus. A similar enzyme is produced by *Vibrio cholerae*. The action of receptor destroying enzyme has been explained by Gottschalk (225) using the results of Blix, Klenk and Kuhn. He proposed the name neuraminidase for the enzyme and defined its action as the hydrolytic cleavage of the glycoside bond joining the keto group of neuraminic acid to D-galactose or D-galactosamine. This also explains why the influenza virus hemagglutinin can be inhibited by other mucoproteins containing neuraminic acid [Schlesinger & Karr (226); Zilliken *et al.* (227); Rosenberg *et al.* (228)]. Gottschalk (229) has recently written a summary of the action of neuraminidase and the enzyme content of other viruses. Jensen (230) has made a comparative summary of the serological relationships among the influenza viruses.

*Newcastle disease and mumps viruses.*—These two viruses can be distinguished from influenza on a morphological basis. In low ionic strength solutions they are almost spherical in form, whereas at higher salt concentrations they have an extended or spermlike shape [Bang (231)]. This behavior is also true for the erythromyeloblastic fowl leucosis virus (232). Newcastle disease virus has also been labeled with  $P^{32}$  using tissue cultures for growing the virus [Franklin *et al.* (233)]. Schmidt-Thannhauser fractionation of the labeled virus showed that some 5 per cent of the  $P^{32}$  was in the RNA fraction but little or no  $P^{32}$  was found in the DNA fraction. A considerable amount of nondialyzable trichloroacetic acid soluble  $P^{32}$  was found. Enzymatic removal of  $P^{32}$  phospholipide from the virus resulted in an inactivation of the particles, demonstrating the essential nature of the phospholipide. Particles of Newcastle disease, mumps and other myxo-viruses were adsorbed on electron microscope films and treated with acid, trypsin and ribonuclease. By this technique trypsin-resistant rings of ribonucleoprotein were detected in the particles (234). As in influenza virus, Newcastle disease virus has non-infecti-

ous hemagglutinating forms which have been studied in detail by Granoff (235). The interaction of Newcastle disease virus with chick embryo fibroblasts was investigated by Levine & Sagik (236). For the attachment anions are necessary, —COOH groups may be involved in this process. The latent period is 3 to 4 hours and the yield per cell 18 to 37 particles. Further details on the adsorption of Newcastle disease virus as well as the early stages of growth have been studied by Rubin & Franklin (237). It is suggested that antibody neutralized virus can absorb to cells but cannot penetrate into them. Growth studies on Newcastle disease virus by Rubin, Franklin & Baluda (238) show that the so-called intracellular infectious virus is actually formed at the cell surface.

#### LARGER ANIMAL VIRUSES

*Tumor viruses.*—Owing to the relationship of the tumor viruses to the general problem of cancer they deserve our special interest. A summary of the tumor viruses has been written by Beard *et al.* (232) and an interesting general survey of cancer biology, including the virus problem by J. Huxley (239). Most important is the Rous sarcoma virus and several other viruses which produce tumors in fowls, since they are the direct etiological agents of the malignant tumors, acting after a latent period of only a few days. This is in contrast to other tumor viruses such as the rabbit papilloma virus and the Bittner milk factor in mice which yield tumors only after month-long latent periods and only in connection with genetic and hormonal factors. Since the purification of these viruses is exceptionally difficult, structural studies have been carried out mostly by the ultrahistology of pathological tissues. Bernhard (240) has written a summary of the work in this field. In 63 per cent of a large number of Rous sarcomas Bernhard *et al.* (241) found characteristic particles, virus-like in nature, with an average diameter of 75  $\mu\mu$ . They have an external membrane and a dense central body with a diameter of 35 to 40  $\mu\mu$ , also surrounded by an inner membrane. Similar particles have been found in other neoplastic material obtained from fowls, for example the Murray-Begg tumor, erythromatosis and myelomatosis, in the last case especially in the mitochondria. Apparently normal tissue contains only a limited number of these particles. Rubin (242) has studied the development of the Rous sarcoma in tissue culture. In contrast to other viruses, the virus production is extremely slow, explaining to some extent the absence of a cytotoxic effect. On the average only one virus is produced per hour per 100 cells. It has often been observed that antiserum to normal chick tissue causes a sharp reduction in the number of virus-induced tumors. Rubin found that the apparent neutralization of Rous sarcoma virus by anti-chick serum was due to impairment of the ability of the infected cells to multiply rather than to a direct neutralization of virus itself. This contradicts some earlier reports which indicated that unaltered normal cell protein forms an integral part of the functional surface of the Rous sarcoma virus. One of the most important developments in this field is the viral induced transformation of normal cells to tumor cells in tissue cultures (243).

In spontaneous mouse mammary carcinoma, virus-like particles have been observed in ultrahistological studies (244, 245, 246). These particles are very similar, if not identical in form to the Bittner milk factor. They have a diameter of 50 to 70  $\mu$  and form inclusion bodies, but are also found singly in the cytoplasm.

*Herpes-viruses.*—In this group belong Herpes simplex, Herpes B, and Virus III as well as the etiological agents of varicella and pseudorabies. In tissue cultures infected with Herpes B two types of particles were demonstrated, those of 60 to 100  $\mu$  diameter and a single membrane, then those of 120 to 180  $\mu$  and a double membrane (247). According to Morgan *et al.* (248) Herpes virus is formed in the following manner. The first visible structure is a primary body 30 to 40  $\mu$  in diameter which later becomes surrounded by a single membrane and finally upon migration from the nucleus into the cytoplasm receives a second membrane.

*Virus of vesicular stomatitis.*—Biophysical studies of this virus by Bradish *et al.* (249) showed that the major part of the infectivity was associated with a component of sedimentation constant 265 S. Electron micrographs of this fraction revealed rods of length 175  $\mu$  and diameter 69  $\mu$ . A component of spherical particles with a diameter of 65  $\mu$  and  $s_{20} = 330$  S was also observed and is probably a noninfectious product of disintegration of the 265 S component. These components constitute about 35 per cent of the total-complement-fixing activity. The remaining 65 per cent was associated with two discrete components of 20 S and 6 S.

*Measles virus.*—Although clinically significant, there is little known about this virus. Ultrafiltration experiments have set the diameter between 100 and 200  $\mu$  (250).

*JHM-virus.*—JHM-virus was discovered by Cheever *et al.* (251) and has been characterized in detail by Drees (252). From ultrafiltration experiments the diameter is 90–130  $\mu$ . The sedimentation constant, determined by bio-assay, lies between 970 and 1200 S.

*Pox-viruses.*—Pox-viruses are brick-shaped in the isolated state, with a longest diameter of 200 to 350  $\mu$ . However, in thin sections of infected cells they are more rounded or ellipsoidal in shape. This is explained by Morgan *et al.* (253) as due to the artificial formation of the brick-like form upon drying. Members of this group are smallpox, vaccinia, cowpox, electromelia, fowlpox, molluscum contagiosum, and rabbit myxoma. Summaries of these viruses have recently appeared (254, 316). A new method for the isolation of vaccinia has been recently described (255). The virus-containing material is homogenized with buffer and a mixture of heptane and fluoro carbons, for example, freon. The aqueous middle layer contains the virus in a state of remarkable freedom from lipides and nonviral proteins. The morphology of the resting vaccinia virus was investigated by Peters (256) by treatment with pepsin, papain, deoxyribonuclease and by sectioning. The virus has a nucleus-like inner body which contains DNA. The external membrane has a double contour. Corresponding to the complicated morphological structure, the chemical composition and antigen structure is also complex. Details can

be found in the above-mentioned review article. Similar studies have been carried out on other viruses of this group.

Advances in the study of the myxoma virus are to be expected with the development of tissue culture techniques for this virus [Charponiere (257)]. The well-known transformation of Shope fibroma into myxoma has been successfully repeated in tissue culture by Kilham (258). The transforming agent was myxoma virus inactivated by heating at 65°C. Positive results were obtained with greater regularity in tissue culture than in rabbits.

*Viruses of the psittacosis group.*—The antigenic structure of this group including the viruses of greatest diameter, has been studied by Benedict & O'Brien (259) and by Ross & Gogolak (260). The properties and chemical nature of the hemagglutinin of the psittacosis group were studied by Gogolak & Ross (261). It contains phospholipide, lecithin and nucleoprotein. Meningo-pneumonitis virus, also belongs to this group, and has been carefully studied by Crocker (262).

#### BACTERIOPHAGES

This discussion is devoted mostly to the T-even phages, since they have been more extensively studied than any other bacterial viruses. The phage structure is similar to that of the other viruses with the DNA responsible for the virus multiplication, located in the interior of the particles, and a protein coat serving to protect the sensitive genetic material and aid in its transmission to bacterial host cells [Hershey (263)]. Rescue of genetic determinants following  $P^{32}$  decay or ultraviolet inactivation and the relationship of inactivation in linked and unlinked determinants provide the best evidence that genetic information is carried by the DNA fraction of T-even coli phages [Herriott (264); Stent & Fuerst (265); Hershey (317)]. The absence of RNA in the phages has been confirmed by Volkin & Astrachan (266) and by Lunan & Sinsheimer (267). The molecular weight of T2 has been studied in detail by Taylor *et al.* (268) and by Bendet *et al.* (269). The sedimentation and diffusion constants are pH dependent. A model has been found which can account for this fact. It is assumed that at pH 5 the particles are tadpole-shaped, but that at pH 7 they project thin filaments laterally from the tail. The molecular weight of about  $200 \times 10^6$  is the same at pH 7 and 5.

*Phage DNA.*—Phage DNA can be separated from the protein coat by osmotic shock or by treatment with chloroform and centrifugation of the denatured protein. Brown & Martin (270) chromatographed the DNA from T2r on columns previously treated with histone and were able to demonstrate thereby that the nucleic acid was not homogeneous. Two fractions could be distinguished by their different base ratios. In 30 per cent of the material the ratio (adenine + thymine)/(guanine + hydroxymethylcytosine) was 1.9 and in 70 per cent it was 2.15. A nonuniformity of the DNA of T2 and T4 is indicated by the experiments of Levinthal & Thomas (271). They have developed a method which is extremely important for the measurement of the radioactivity of single particles. If a particle containing a  $\beta$ -emitting isotope

is embedded in a nuclear emulsion and the exposure time is adjusted so that several disintegrations occur before development, then one can observe a number of electron tracks. These groups of tracks are called stars. The molecular weight of the star-forming particles can be determined from the average number of rays per star, if the chemical composition of the star-forming particle is known. T2 and T4 have a piece of DNA of average molecular weight between  $19-27 \times 10^6$  and several (six or more) small pieces of DNA. The large piece contains 40 per cent of the entire P in the phage and the other 60 per cent must be in the smaller pieces, which are too small to form stars. How close a connection exists between the chromatographic and radiographic results can only be determined by further experimentation. A further technique which could be very significant in the differentiation of phage DNA has been developed by Meselson, Stahl & Vinograd (272). They have succeeded in separating phage DNA labeled with 5-bromouracil from normal phage DNA by sedimentation in a continuous density gradient, established by the sedimentation of a low molecular weight solute. The presence of glucose in the DNA of even-numbered phages was discovered by Sinsheimer (273). The glucose was shown to be a glucoside substituent of the hydroxymethyl groups of hydroxymethylcytosine by Volkin (274). Jesaitis (275) studied the DNA of the wild type strains of T2, T4 and T6 phages. The amount of hexose present in each nucleic acid differs significantly. In the case of T6 nucleic acid two molecules of glucose in the form of a diglucoside are linked to hydroxymethylcytidylic acid. T2 contains both free hydroxymethylcytidylic acid and its monoglucoside and T4 only the monoglucoside. Sinsheimer (276) has also showed that in wild type T2 23 per cent of the hydroxymethylcytosine is not substituted with glucose, while in T4 all the hydroxymethylcytosine is glucose substituted. The inheritance of this glucose property seems to be linked with the bar properties of the phages [Streisinger & Weigle (277)]. The interpretation of the results is complicated by the inhomogeneity of the DNA. Cohen (278) has presented data which suggests a relationship between the glucose content of the phage DNA and the r-property, but the data presented by Sinsheimer do not support this relationship.

**Biosynthesis.**—Treatment of infected cells with various inhibitors has been used to obtain a clearer picture of phage synthesis. Mustard gas inactivated T2h phages can be restored to infectivity by multiplicity or cross-reactivation [Papirmeister *et al.* (279)]. Hence the mustard gas probably acts on the DNA to destroy certain markers which control the injection of the nucleic acid into the host cell. 7-Azatriptophane inhibits the formation of T2 phage but does not kill the noninfected bacterial cells [Pardee *et al.* (280)]. Azatriptophane is probably incorporated into the phage protein resulting in the production of nonviable phages. If proflavin is present during phage infection, little or no infectious progeny are liberated, but the infected cells synthesize DNA at about the same rapid rate observed in the absence of proflavin. The DNA may be phage DNA since it contains hydroxymethyl-

cytosine instead of cytosine [de Mars (281)]. Using a method of general interest, Astrachan & Volkin (282) found that there was a slight difference in the polynucleotide sequence of normal phage DNA and the DNA synthesized in the presence of proflavin. The action of chloramphenicol on the phage DNA synthesis has been studied in several laboratories. According to Tomizawa & Sunakawa (283), the increase of DNA in phage-infected bacteria was completely suppressed by the addition of chloramphenicol within 2 min. following infection. Addition after the 10th min. showed no appreciable effect on DNA synthesis, despite cessation of intracellular phage formation and protein synthesis. It is concluded that protein synthesis is necessary to initiate DNA synthesis, but not for its continuation, a conclusion supported by the studies of Burton (284) and Crawford (285), and further elucidated by the work of Hershey & Melechen (286). If chloramphenicol is added to the bacterial culture 7 to 11 min. after infection, synthesis of DNA proceeds with very little concomitant protein synthesis. If chloramphenicol is subsequently removed, phage particles promptly start to form and the nucleic acid synthesized during the chloramphenicol period enters into them. The bulk of the phage precursor nucleic acid is not formed inside particles possessing a protein membrane.

Interesting studies have been made by Green & Cohen (287a to 287d) on the synthesis of the pyrimidine components of phage DNA. Using a uracil-requiring strain of *Escherichia coli*, it was shown that all pyrimidine components of the phage are formed from the uracil of the external medium. The methyl group of thymine originates from glucose; the methyl group of methionine and the hydroxyl group of 5-hydroxymethylhomocysteine were not utilized for the biosynthesis of thymine and hydroxymethylcytosine of T6r<sup>+</sup> phage. Further details of the biosynthesis of the nucleic acids have been discussed by Cohen (288). Volkin & Astrachan (289) demonstrated that in T2 infected *E. coli* some metabolic activity of RNA occurs despite the absence of net RNA syntheses. It was noted that at very short time intervals after addition of P<sup>32</sup> the amount of isotope accumulated into RNA was at least as great as that found in DNA. These results show that phage DNA injected into the bacterial cell does not synthesize the phage protein directly, but that there is a complicated interaction between RNA, protein, and DNA synthesis. This process needs further clarification. This concept was further supported by the recent study of Spizizen (290) on the infection of bacterial protoplasts by T2. Protein-free DNA isolated from the virus could not produce infective particles, but DNA units from disrupted phages were involved in the virus synthesis. However, Fraser *et al.*, working with protoplasts, presented evidence that the actual infection occurs with DNA (318).

**Protein coat.**—The protein coat of the T-even phages has a complicated structure. The following components can be distinguished. (a) The protein which serves as the coat of the phage head; (b) a plug lying within the phage tail, the shell of which is composed of at least two structures; (c) the distal



cover; (d) the proximal cover; (e) an enzyme which acts on the membrane of the host cell; and (f) an acceptor which can react with the specific receptor. It is still open to question whether the enzymatic activity and the acceptor property can be correlated with the distal and proximal cover. The distal half of the tail protein of T2 can be removed by the specific action of complexes of the zinc group metals. After this treatment the phages are very labile and the DNA can easily be released by various agents [Kozloff & Henderson (291)]. The same effect can be obtained by the use of oxidizing agents [Kellenberger *et al.* (292)]. The projecting plug remains unaltered, even after extensive oxidation. The distal end of the tail seems to be composed of three fibers which can be made visible by repeated cycles of freeze drying and thawing (293). After this procedure, the phage DNA could no longer be injected into the bacteria. Oxidized T2 is still adsorbed to host cells, but oxidized T4 cannot adsorb. The production of phage related structures during multiplication of T2 and T4 was studied by Kellenberger and co-workers (294, 295). The intracellular appearance of rods and empty heads is not influenced by the presence or absence of proflavine.

The protein coats which remain after the removal of the DNA still possess several of the biological functions of the original virus (319). For example, they can still cause bacterial lysis [Jerriott & Barlow (296)]. Further studies by French & Siminovitch (297) showed that only 10 to 35 per cent of the ghosts are bacterial killers. The nonkilling ghosts have an interesting temporary effect on the bacteria: inhibition of protein synthesis plus exclusion of T2 and T1 by T2 ghosts. This action is reversed in 50 min. The action of the phage enzyme on isolated host cell walls has been studied by Barrington & Kozloff (298), Brown & Kozloff (299) and Koch & Weidel (300). The enzyme is located on the tip of the tail and splits protein structures from the bacteria as well as from the isolated bacterial membranes. The enzymatically active protein is of low molecular weight. The enzyme has been concentrated and partially purified from lysates of *E. coli* infected with T2 by Koch & Jordan (301) and its action was further studied (320). It is not yet certain that the splitting off of a chemically defined substance from bacterial membranes, the killing of bacteria, and the lysis of bacteria are functions of the same agent. In the steps used for concentration and purification, however, the three activities always remained together in one fraction.

The phage receptor sites on the cell membrane are not attacked by this enzyme. The T2 receptor substance on the bacterial wall has not yet been purified, but that for T5 has been purified and characterized [Weidel and co-workers (302, 303, 304)]. This is a spherical particle of 31  $m\mu$  diameter and composed of a lipoglycoprotein. A molecular weight of  $12 \times 10^6$  has been determined by physico-chemical methods. Nothing certain can be said of the nature of the corresponding acceptors on the tip of the phage tail. It is possible that neutralizing antibody combines to these acceptors and that the acceptors are responsible for the so-called serum blocking power (281). This, however, is contradicted by the fact that serum neutralized phage still ad-



sorbs to host cells but in a reversible fashion [Nagano (305)], only the irreversible adsorption is inhibited by the antiserum. The adsorption process of phages is discussed in more detail in a review by Weidel (306).

Little is known concerning the chemical composition of the individual protein components. A comparison of the amino acid composition of T2 and T3 bacteriophages was carried out by Fraser (321). End-groups analyses on shocked T2r<sup>+</sup> showed that alanine was the only N-terminal group, 1 mole per peptide chain of molecular weight 80,000. It is not yet clear to which protein component this end-group belongs (307). Studies on other groups of phages cannot be discussed in this review.

#### ACKNOWLEDGMENT

The author is greatly indebted to Dr. M. Wiedemann for the help in preparing this manuscript and Dr. R. M. Franklin for the translation.

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## BIOCHEMISTRY OF CANCER<sup>1,2</sup>

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Over the past decade authors faced with the responsibility of writing this chapter have made reference to the breadth and magnitude of their undertaking. We join our predecessors in wondering how to do justice to our assigned task in the assigned space. The literature concerned with the biochemistry of cancer, we expect, will continue to be almost as broad as the field of biochemistry until the scientific community has been convinced of the discovery of the site(s) of the biochemical lesion(s) responsible for and associated with neoplasia.

For this reason, as well as in compliance with the request of the editors that the review not take the form of a bibliographic recitation, we have drastically limited the scope of the present effort. To those who venture to read this limited review it will become apparent that it is concerned in the main with recent biochemical and related literature associated with certain fundamental aspects of cancer chemotherapy (1). Reference will be made to reviews that have appeared during the past year which, it is hoped, will make up for our numerous omissions.

### BIOCHEMICAL DIFFERENCES BETWEEN NORMAL AND NEOPLASTIC TISSUES

It is difficult for the biochemist of this era to concede that the profound behavioral differences between neoplastic cells and their normal cells of origin are not associated with significant metabolic differences of a qualitative or quantitative nature. The practical motivation for much research in this area resides in the conviction that knowledge of biochemical transformations preferentially employed by or peculiar to cancer cells may serve as a guide to selection or synthesis of antimetabolites or other classes of chemicals with greater chemotherapeutic indices.

These searches for exploitable biochemical differences between normal and neoplastic cells are vastly complicated by the lack of unequivocally satisfactory experimental systems, be they *in vivo*, tissue culture, tissue slices or homogenates, or partially purified enzymes. Possibly the immense

<sup>1</sup> The survey of the literature pertaining to this review was completed in September, 1957.

<sup>2</sup> The following abbreviations are used in this chapter: ADP for adenosine diphosphate; AMP for adenosine monophosphate; APP for 4-aminopyrazolo(3, 4-d) pyrimidine; ATP for adenosine triphosphate; DNA for deoxyribonucleic acid; GTP for guanosine triphosphate; Pi for inorganic phosphate; P-P for pyrophosphate; RNA for ribonucleic acid; TPN for triphosphopyridine nucleotide; and TPNH for the reduced form of triphosphopyridine nucleotide.

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strides which have been and are being made in tissue culture propagation of mammalian cells will in time help to overcome this deficiency that has so long plagued the biochemist and filled the literature with controversial data regarding differences between "normal" and "neoplastic" tissues. However, there is at present considerable indecision as to the normalcy of "normal" cells growing in tissue culture and this seemingly ideal approach to providing normal and neoplastic cells growing at the same rates *in vitro* would appear to lack the host-tumor association which looms so important in the minds of many competent biologists. Busch *et al.* have recently commented (2, 3):

In view of the fact that growth and survival of tumor cells are of primary interest because of their effect in the whole animal, it would seem necessary in future studies to recognize the significance of *in vitro* studies as indicating "capacity" of tumor tissues in certain metabolic pathways and of *in vivo* studies as indicating the "activity" of tumor tissues in the natural environment of tumor cells.

Greenstein in a similar vein has stated (4):

It is possible that studies of cellular metabolism, which began with observations on the whole animal and then progressed successively through studies of isolated organs, tissue slices, homogenates, cell fractions, and finally highly purified individual metabolic factors can with profit turn back to the whole animal. Studies of the effect of constitutional factors on metabolic reactions of tumors *in vitro* provide a certain interest but, like all *in vitro* approaches, are at the mercy of the experimental conditions which the investigator chooses to select.

In searches for biochemical differences between normal and neoplastic cells it is the "control"—the tremendous variety of normal tissues and large array of cell types in each normal tissue, each with its characteristic metabolic pattern—that so complicates the problem. In all probability an exploitable biochemical difference, from the standpoint of cancer chemotherapy, must be an obligate biochemical event, insofar as certain neoplastic cells are concerned, but one that is unimportant under the conditions employed to perhaps scores of types of vital normal cells. In the light of present knowledge, it seems likely that such an exploitable biochemical difference may have to be more than a rate difference associated with cellular biosynthesis of gene chemicals and protein, because there are normal cells such as intestinal epithelium and bone marrow that are equal to the most malignant neoplasm in mitotic activity and capacity to synthesize DNA, RNA, and protein and that do not encroach on other tissues because of sloughing or cellular expulsion mechanisms.

One does not find many hypotheses, clearly stated as such, concerning the possible site of the biochemical lesions which are responsible for cancer. This is perhaps because scientists are seldom criticized for theorizing on relatively obscure issues; the criticism awarded a given hypothesis is apparently more or less proportional to the importance of the issue being considered. Hypotheses on possible altered biochemical areas or cellular function that some believe might be associated with cancer are more prevalent. Certain of these

which have received recent consideration will be accorded brief comment herein.

**Glycolysis.**—The Warburg theory, which proposes that cancer results when a normal cell adopts an anaerobic metabolism as a means of survival after injury to its respiratory mechanisms, was proposed some thirty years ago and has been and still is the subject of considerable research and controversy. The status of this controversy has been reviewed most recently at a symposium sponsored by the American Cancer Society (5).

The following objections to the theory that oxidative metabolism in tumors is impaired were raised (6):

(a) Despite their high glycolysis, oxygen consumption in tumors is not quantitatively diminished; in general, a representative group of tumors absorbs oxygen about as rapidly as a comparable group of normal tissues.

(b) Although tumors produce large amounts of lactic acid, so do many normal tissues.

(c) Tracer studies *in vitro* have demonstrated that various tumors convert glucose and fatty acids to carbon dioxide at rates similar to those exhibited by normal tissues; this is indeed strong evidence of an operative respiratory metabolism in neoplastic tissues.

(d) Published data indicate that oxidative phosphorylation occurs in tumor mitochondria as it does in mitochondria of normal cells.

Weinhouse has suggested that high glycolysis occurs "despite" quantitatively and qualitatively normal occurrence of carbon and electron transport and further suggested "that glucose catabolism is so rapid in tumors that normal channels for disposal of pyruvate are overloaded" (6).

Harking back to the above-mentioned lack of ideal biological systems for the study of biochemical differences between normal and neoplastic cells, the critics of the Warburg theory are still faced with the interesting results, *in vivo*, of Potter & Busch (7) which suggested that tumors were unable to convert fluoroacetate to fluorocitrate. Most normal tissues of fluoroacetate-treated rats accumulated citrate whereas the tumors did not. More recent data, *in vivo*, of Busch & Baltrush (8) showed the half-time of disappearance of acetate-1- $C^{14}$  of tumor tissues was about 6 to 45 times that of various normal tissues.

It has also been noted\* in experiments, *in vivo*, that pyruvate-2- $C^{14}$  is converted extensively to the amino acids—alanine, glutamic acid, and aspartic acid—as well as lactic acid in nontumor tissues, whereas in tumors, amination reactions were not significant (9).

Busch *et al.*, in an effort to bridge the gap between the studies, *in vitro*, which have demonstrated the existence of citric acid cycle enzymes in tumors and the *in vivo* data which show a diminished activity of tumors in oxidizing labeled acetate and accumulation of citrate in tumors of fluoroacetate-treated animals, have observed that "tumors have the capacity under certain conditions to form citrate *in vitro* in the presence of excess substrate and high atmospheric oxygen tension." It was postulated that the lack of activity of



tumors *in vivo* in the formation of citrate in fluoroacetate-treated animals might be a consequence of low oxygen tension associated with a lack of essential substrates and reduced intratumor pH (2).

In the opinion of the reviewers, there is still lacking any compelling evidence that a "faulty" or "overloaded" citric acid metabolism is the primary area of biochemical alteration responsible for neoplasia; however, if further studies *in vivo* can specify the initial events responsible for reduced acetate catabolism, *in vivo*, their exact nature, and their over-all metabolic consequence, the status of the biochemistry of cancer will indeed have been advanced. It is hardly necessary to re-emphasize at this point the obvious interrelationships between glycolytic and respiratory events, nucleotide and nucleic acid metabolism, amino acid metabolism, fatty acid metabolism, oxidative phosphorylation, electron transport, or in fact, the apparent interdependence of all biochemical events which arbitrarily are considered somewhat separately in the minds of men but operate as a unit insofar as nature is concerned.

In a search for compounds which might selectively inhibit the enzymes essential for function and growth of neoplastic tissue some attention now has been turned to specific inhibitors of glycolysis (10). The only agents found to date that inhibit lactic acid dehydrogenase selectively are oxamic acid, oxalic acid, and fluoropyruvic acid. Concentrations of fluoropyruvic acid which suppressed lactic acid formation by Walker 256 carcinosarcoma slices to about 50 per cent had little effect on the oxygen uptake of tumor, liver, or kidney slices. Studies such as these are of especial value from the point of view of those interested in chemotherapy and from the mechanistic standpoint of dissecting glycolytic and oxidative biochemical events. One has but to recall the value of inhibitors such as fluoroacetate, sulfonamide, aminopterin, and azaserine, to mention a few, in establishing firm biochemical knowledge, to wax enthusiastic over efforts to uncover selective enzyme inhibitors.

Other papers of interest and pertinence to the problem of glycolytic or oxidative differences between normal and neoplastic tissues have appeared during the past year (11 to 47).

*Nucleotide and nucleic acid metabolism.*—The rationale underlying searches for exploitable biochemical differences between normal and neoplastic cells in the area of nucleotide metabolism stems largely from two considerations:

(a) Nucleic acids are presumably major constituents of two biological entities, genes and viruses, which are most frequently associated with the "neoplastic change."

(b) Many of the temporarily effective anticancer agents have been shown to affect nucleotide metabolism; i.e., inhibitors of purine and thymine synthesis, *de novo*, and certain analogues of hypoxanthine, adenine, guanine, orotic acid, uracil, and cytosine. Since such agents possess a demonstrable chemotherapeutic index in cancer-bearing mammals, it is tempting to as-

sume that biochemical differences between host and neoplastic tissues exist somewhere in the area of nucleotide metabolism. It is not yet settled as to whether the presently known anticancer agents exert their primary action at the cofactor or the nucleic acid level. However, it appears quite clear that the rates of DNA synthesis and mitotic activity of cells are parallel (48 to 51). Thus, any agent or set of experimental conditions selected, that inhibits DNA synthesis, may be expected to inhibit division of living cells.

Cohen (52), in well-designed model experiments, has demonstrated that a most effective means for "killing" bacterial cells is to provide conditions under which RNA and protein synthesis proceed but DNA synthesis is blocked. Under such conditions bacterial cells enlarge in size, fail to divide, and die; hence, in the words of Cohen, these cells "must metabolize and grow in order to die." Any rational extension of this concept to cancer chemotherapy would appear to require knowledge of differences in the biochemical events leading to polynucleotide synthesis in normal and neoplastic cells.

*Purine biosynthesis.*—Labeled compounds (glycine, formate, 4-amino-5-imidazolecarboxamide) which, in mammalian cells, are or can readily be converted to purine precursors are incorporated into DNA and RNA purines of cancer cells and mitotically active normal cells at rates that are of the same order (53, 54, 55). In addition, when a labeled precursor, formate, is administered to cancer-bearing animals, similar intermediates are observed on chromatogram-autoradiograms prepared from extracts of cancer or normal tissues: formylglycinamide riboside, 5-amino-4-imidazolecarboxamide ribotide, inosine-5'-phosphate, adenosine-5'-phosphate, guanosine-5'-phosphate, and di- and triphosphates of adenosine and guanosine (56). Tomisek has demonstrated that amethopterin causes an accumulation of 5-amino-4-imidazolecarboxamide ribotide with concomitant inhibition of purine nucleotide formation in both normal and leukemic cells (56). Moore & LePage have observed accumulation of formylglycinamide ribotide in azaserine-inhibited normal and neoplastic mammalian cells (57), thus indicating a site of blockade similar to that observed in pigeon liver and *E. coli* (58, 59).

For reference in this section and also in the following sections, the *de novo* route to purines, as currently conceived, is given in Figure 1, which also shows the sites of action of certain agents to be discussed later. The abbreviations used in this chart will be used throughout the remainder of this chapter. The scheme shown is that given by Buchanan (95); the metabolic blocks have been inserted by the authors of this review.

The above-mentioned observations and many others suggest that the intermediates en route to purine nucleotides may be qualitatively similar in various normal and neoplastic cells.

Large quantitative differences in the incorporation of labeled purines and their derivatives into the polynucleotides of normal and tumor tissues have been observed in experiments carried out *in vivo* (53, 54, 55, 60). Guanine particularly has been shown, on comparison with the host's normal tissues, to be incorporated poorly into the nucleic acids of neoplasms (mouse,



rat, and human) growing in rodents. This poor incorporation of guanine *in vivo* into tumor nucleic acids may be overcome by pretreatment of the host with 4-amino-5-imidazolecarboxamide, an inhibitor of guanase (61). Balis has observed that tumor *breis* incorporate guanine quite effectively when compared with liver *breis* (62) and has suggested that the poor utilization by tumors *in vivo* may be dependent on the amount of labeled guanine which reaches the tumor. Here again differences between results *in vivo* and *in vitro* must be resolved with cognizance of the import of the former to the problem of chemotherapy.

The rates of DNA synthesis and cell division have now been shown to be parallel in bacterial cells (48, 49), mouse Strain L cells growing in tissue culture (51), and Ehrlich ascites tumor cells growing in the peritoneal cavity of mice (50). RNA synthesis paralleled mitotic activity in some of these experiments. A general relationship between rates of polynucleotide synthesis and mitotic activity of animal tissues has been repeatedly observed over the past decade. Synthesis of DNA *de novo* in mitotically inactive tissues such as adult (resting) liver is minimal when compared to regenerating liver or rapidly dividing neoplastic or normal cells. If, by chance, the biosynthesis of a full complement of polynucleotides were associated with mitosis, it would be of considerable importance to ascertain why nondividing cells such as those of the liver, which synthesize purine nucleotides quite actively (63), fail to polymerize these nucleotides. Is it because such resting cells lack polymerization capacity (regenerating liver has this capacity in abundance) or is high nucleotide-degrading capacity associated with minimal nucleic acid synthesis and cell division (53)?

De Lamirande (64) has found that Novikoff hepatoma (one of the most rapidly growing experimental neoplasms) is quite low in certain nucleoside- and purine-degrading enzymes when compared to adult rat liver. This liver tumor is apparently devoid of xanthine oxidase.

Lewin *et al.* have reported that xanthine oxidase is highest in breast tissue of C Strain mice (low tumor strain), significantly lower in breast tissue of mice with the "Bittner factor" (high tumor strain), and lower still in spontaneous mammary tumor tissue (65). Bergel *et al.* have put forth the hypothesis that xanthine oxidase may be the key enzyme in control of cellular purine pools and mitotic activity (66).

**Pyrimidine biosynthesis.**—Recent results on the comparative utilization of labeled precursors of nucleic acid pyrimidines by normal tissues and neoplastic tissues growing in the intact animal have been reported by Eidinoff *et al.* (67) and Heidelberger *et al.* (68). When  $C^{14}$ -labeled ureidosuccinic acid was administered to rats bearing human sarcoma transplants (HS-1) and the specific activities of nucleic acid pyrimidines were measured at seven hours, the order of decreasing specific activity for thymine was tumor, intestine, spleen, and liver. The specific activity of DNA cytosine was equal to or greater than DNA thymine. The liver RNA pyrimidines had the highest specific activity of the tissues studied, with uracil approximately twice as active as cytosine (67).

When C<sup>14</sup>-labeled uracil was administered to rats bearing the Flexner-Jobling carcinoma, this pyrimidine was utilized for acid-soluble nucleotide formation and RNA and DNA pyrimidine biosynthesis to a greater extent by the carcinoma and intestinal mucosa than by liver. Labeled orotic acid was utilized more efficiently than uracil by intestinal mucosa but not by the tumor. It was pointed out that in soluble fractions from homogenates of liver and tumor there was extensive catabolism of both uracil and dihydro-uracil in the liver system but very little in the tumor system (68).

From the data now at hand, it would appear that no profound differences in anabolic pathways leading to pyrimidine nucleotides or polynucleotides have been observed in normal and neoplastic tissues.

Schneider & Brownell have observed that the concentration of acid-soluble deoxyriboside compounds in the liver increased more than 60 per cent following partial hepatectomy. This increase in deoxyriboside compounds in the liver occurred before the rapid cell division of the regenerating liver, suggesting that such compounds represent DNA precursors (69). When normal rat liver and Novikoff hepatoma were compared, it was noted that in the former deoxycytidine accounted for almost all of the deoxyriboside compounds, whereas the hepatoma contained deoxycytidine, deoxyuridine, and thymidine in an approximate ratio of 5:1:1 (70). LePage found no evidence of the presence of deoxyadenosine monophosphates in extracts of rat tissues including Flexner-Jobling carcinoma (71).

Thomson *et al.* (72, 73) have observed that Ehrlich ascites carcinoma cells of the mouse virtually lose ability to carry out DNA purine synthesis *de novo* in tissue culture, although formate incorporation into DNA thymine remained high. Polynucleotide purine synthesis could be restored to such cells in tissue culture by addition of extracts of liver, spleen, or muscle.

*Growth requirements of mammalian cells in tissue culture.*—Another promising approach to discovery of biochemical differences between normal and neoplastic cells is the study of minimal requirements for growth and cell division of representative cell types (normal and neoplastic) in tissue culture media of known composition.

Eagle *et al.* (74) has reported that

if embryonic, adult liver, and conjunctival lines here studied are still "normal" cells (and there appears to be no presently available definite criterion short of test in man), one must then conclude that malignant cells do not regularly differ from normal cells with respect to the exogenous amino acids needed for survival and growth, and the amounts required for maximal growth.

The seven lines of cells ("normal" and neoplastic) studied by Eagle required arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, and valine.

To date, seven vitamins have proved essential for survival and multiplication of a mouse fibroblast (Strain L) and a human carcinoma cell (HeLa) in tissue culture: choline, folic acid, nicotinamide, pantothenic acid, pyridoxal,

riboflavin, and thiamine. "The maximally effective concentrations were in the range  $10^{-7}$  to  $10^{-8}$  gm. per ml." (75).

Evans *et al.* (76) have reported that mouse cells of Strain 929 clone L will proliferate in protein-free chemically defined media; proliferation was generally greater when vitamin B<sub>12</sub> was added to the medium. Some evidence was presented that several coenzymes and deoxyribosides could not be omitted from the media without adverse effect on population increase. Addition of horse serum and chick embryo extract, or as little as 10 mg. per cent of gross globulin of horse serum, to the chemically defined medium substantially increased the rate of cellular proliferation, which indicates that considerable effort is still required before optimal tissue culture growth can be obtained in a completely synthetic medium.

*General theories.*—Osgood, on the etiology of neoplasia, has recently presented a general hypothesis based on biologic and morphologic considerations. He has postulated that the "fundamental alteration" is any genetic change which leads to lack of any enzyme system necessary for a full life span of the differentiating cell of a series.

This chemically mature differentiating cell produces inhibitors of cell division. Lack of the inhibitors permits the emergence of the capacity for unlimited and uncontrolled growth that is present in the unicellular organisms and has been retained in the early dividing cells of every fundamental cell type and at least some stage of postnatal life (77).

This concept would be more convincing if one could point to direct experimental proof of production of some characterized growth inhibitor by mature differentiating cells.

Thus we stand today with reported experimental results regarding nucleotide and nucleic acid metabolism which suggest that:

(a) The *de novo* pathway leading to the major nucleotide purines and nucleic acid purines is qualitatively similar in normal and neoplastic cells. Labeled compounds which find their way into this series of biochemical events at various points proceed through numerous transformations to the gene chemicals of both normal and cancer cells. The rates of these events are proportional to the rates of cell division in normal and neoplastic tissues, and the rate-controlling factors are yet to be elucidated.

(b) On blockade of purine synthesis, *de novo*, similar metabolites accumulate in both normal and neoplastic cells.

(c) For reasons not yet understood, certain "preformed" purines or derivatives are poorly incorporated, relative to the host normal tissues, into the nucleic acids of experimental neoplasms growing in the intact animal.

(d) Both *de novo* precursors and preformed pyrimidines and derivatives are incorporated into the nucleic acids of dividing normal and neoplastic cells at rates which are of the same order. Qualitative differences in pyrimidine nucleotide and polynucleotide synthesis of normal and neoplastic cells have not been reported.



(e) The metabolic requirements of neoplastic and "normal" cells growing in tissue culture are (as far as is known) similar.

(f) There is some evidence that the purine and purine nucleoside catabolizing capacity of rapidly dividing neoplastic cells is significantly lower than that of a nondividing tissue such as liver or normal breast tissue.

#### MECHANISM OF ACTION OF AGENTS WITH TEMPORARY ANTICANCER ACTIVITY

A number of agents are known that have profound, but temporary, inhibitory activity against one or more types of experimental tumors. Each year sees the addition of new members to this list and considerable activity in the study of mechanisms of action of agents, both old and new. Although much has been learned of the mechanism of action of some agents, there is as yet no complete explanation of the anticancer action of any agent, in the sense that such an explanation should include not only a precise definition of the metabolic site of action, but also an understanding of the biochemical factors responsible for selective toxicity to cancer cells.

Inconsistencies in results obtained in different biological systems have been discussed above as a factor militating against interpretations and correlation of results of studies on biochemical differences; the same considerations apply also to mechanism studies, which have been carried out in a great variety of systems. The present state of knowledge is not yet such that any single system can with surety be selected for a study of the mechanism of an agent; rather it would appear desirable to study each agent in a number of systems since the total of information gained in several systems may represent a fuller picture of its mechanism than can be obtained from studies confined to a single system.

A vast amount of literature has accumulated in recent years on the mechanism of action of anticancer agents, and a complete and critical review of each of the better known agents is therefore obviously beyond the scope of this review. As an alternative, an attempt has been made to discuss only the recent literature on mechanism and to correlate it with the older literature on the subject as it has been summarized in the many reviews that have appeared in the last few years.

*Amethopterin and other antifolics.*—A number of agents are known that interfere with folic acid metabolism; of these, amethopterin (4-amino N<sup>10</sup>-methylpteroylglutamic acid) has been the subject of most of the recent mechanism study. Antifolics presumably interfere with the formation of coenzymes F which are cofactors for a number of reactions involving one-carbon metabolism. These reactions include the *de novo* synthesis of purines and of thymine; inhibition of one or both of these syntheses has been presumed to be the mechanism by which antifolics inhibit growth (78). Although there is no evidence from mammalian systems to indicate one or the other as the primary site of inhibition, the results obtained by Cohen (79) in bacteria (already mentioned above) may well be pertinent. In bacteria that synthesize folic acid, sulfa drugs which inhibit synthesis of folic acid from its

precursors apparently have the same metabolic effects as do antifolics in mammalian cells. Cohen found that bacterial cells, treated with sulfa and supplied with purines and the other products of one-carbon metabolism except thymine, continued to synthesize RNA and protein while DNA was inhibited and the cells died as a result of this imbalanced growth. Cohen has postulated that many anticancer agents may kill cancer cells by a similar selective inhibition of DNA synthesis. Until recently, the means of testing this concept in mammalian systems have not been available; however, the variety of cells now available in tissue culture provide apparently suitable systems for such study.

Most of the recent work on antifolics has done little to change earlier concepts of mechanism and represents essentially refinements or extensions of observations to other systems. Tumor-bearing animals, when treated with amethopterin, accumulated the ribotide of 5-amino-4-imidazolecarboxamide and the corresponding riboside in both tumors and normal tissues (80, 81), substantiating the fact that in mammals amethopterin blocks purine synthesis at the same site that sulfa drugs have long been known to block in bacteria (82). In pigeon liver extract, aminopterin (4-aminopteroylglutamic acid) blocked the conversion of 4-amino-5-imidazolecarboxamide to purines (83). Studies with bone marrow *in vitro* showed that amethopterin prevented the methylation of uracil deoxyriboside (84). Hakala (85) found that amethopterin inhibited growth of Sarcoma 180 in tissue culture and that inhibition could be prevented by the presence of hypoxanthine, thymidine, and glycine. These observations are all consistent with the sites of blockade established earlier.

The effects of amethopterin on purine synthesis *de novo* may be determined by measuring its effects on incorporation of formate or glycine in nucleic acid purines. In attempts to relate variations in clinical response to variation in effectiveness of amethopterin on purine biosynthesis, Winzler and co-workers (86, 87) have studied its effect on incorporation of formate and glycine into nucleic acids by human leukocytes *in vitro*. Significant differences were noted in the uptake of formate by different types of human leukocytes and the effects of amethopterin thereon. These results do not necessarily prove purine synthesis as the metabolic area primarily responsible for variations in response of leukemias to amethopterin since the possibility exists that inhibition of thymine synthesis may be the primary site of action.

Formiminoglutamic acid has been found in the urine of leukemic children treated with amethopterin; this probably indicates that folic acid derivatives are involved in the transfer of formimino groups in man (88, 89). The observation that amethopterin increases the level of liver glutathione in leukemic animals has been interpreted as indicating an increased demand for glutathione by the tumor and a consequent increased output by the liver, which results in an accumulation in the liver when the tumor is inhibited by amethopterin (90).

Antifolic activity is not restricted to pteridines; certain 2,4-diaminopy-

rimidines and 1,2-dihydrotriazines have been known for some time to possess antifolic activity (91). Two new series of compounds have been studied by Timmis *et al.* (92) and by Modest, Schlein & Foley (92a, 92b) as folic acid antagonists: these are 5-arylazopyrimidines and 8-aryl-8-azapurines each with amino groups on the pyrimidine ring. The discovery of activity in these series probably does not provide any new agents of clinical usefulness since the most active member had only one-fiftieth the activity of amethopterin toward *Streptococcus faecalis*; however, the structure-activity relationships observed among the large numbers of compounds screened are of interest.

*Azaserine and 6-diazo-5-oxo-L-norleucine.*—Azaserine (O-diazoacetyl-L-serine) and the related compound, 6-diazo-5-oxo-L-norleucine, have been found to act similarly on a number of systems and will be considered together here. Azaserine, the first of these compounds to be discovered, was early found to inhibit *de novo* purine synthesis (93) and diazo-oxo-norleucine was shown to have the same effect, though active at a much lower dose level (58, 94, 95). In a pigeon-liver enzyme system, azaserine and diazo-oxo-norleucine were found to act specifically as glutamine antagonists in inhibiting the conversion of formylglycinamide ribotide to formylglycinamidine ribotide (58) (Figure 1). In azaserine-inhibited *Escherichia coli*, formylglycinamide ribotide and the corresponding riboside accumulate with concomitant decrease in purine synthesis *de novo* (59).

The effects of azaserine on the incorporation of a number of purine precursors *in vivo* are consistent with this site of blockade (96). Studies *in vivo* with tumor-bearing animals have shown that azaserine and diazo-oxo-norleucine cause an accumulation of formylglycinamide ribotide and, in some tissues, an unknown intermediate; when higher doses of the inhibitor are given, no accumulation of the ribotide occurred (57). Since it has been shown that in pigeon liver extract diazo-oxo-norleucine inhibits the synthesis of glycinamide ribotide (94), probably by interfering with the synthesis of phosphoribosylamine, a glutamine-requiring reaction, this result would suggest that *in vivo* the conversion of formylglycinamide ribotide to formylglycinamidine ribotide is the most sensitive to inhibition but that at higher levels inhibition of less sensitive reactions may become of importance.

LePage and co-workers have obtained a correlation between the inhibition of *de novo* purine synthesis by azaserine and the effects of the latter in inhibiting growth of solid tumors (97). Azaserine and diazo-oxo-norleucine were found to react irreversibly with the enzyme involved in conversion of the ribotide of formylglycinamide to that of formylglycinamidine, and inhibition could be prevented by prior administration of glutamine but could not be reversed by subsequent addition (58, 98).

Glutamine is also involved in other conversions along the *de novo* pathway to purines, namely, the formation of phosphoribosylamine and the conversion of xanthosine-5'-phosphate to guanosine-5'-phosphate. Azaserine has been found to inhibit both of these reactions *in vitro* (98a, 98b).

There is thus considerable evidence for purine synthesis as the primary site of blockade by azaserine and diazo-oxo-norleucine. One other site of

action has been definitely established in the nucleic acid area: both azaserine and diazo-oxo-norleucine have a specific effect *in vitro* and *in vivo* on cytosine synthesis and this has been shown to occur at the stage at which uracil derivatives are aminated (99).

The above references to mammalian systems *in vivo* and *in vitro* implicate nucleic acid synthesis as a primary site of attack by azaserine and diazo-oxo-norleucine and emphasize the similarities in action between these agents. In other biological systems, other metabolic areas become of importance and differences between the effects of azaserine and diazo-oxo-norleucine become apparent. In *E. coli*, azaserine inhibition was most effectively reversed by the aromatic amino acids, and less effectively by purines, 4-amino-5-imidazolecarboxamide, and methionine (96, 100). However, inhibition induced by diazo-oxo-norleucine was not reversed by aromatic amino acids, but was effectively reversed by purines and purine derivatives; furthermore, in this system, cross-resistance between diazo-oxo-norleucine and azaserine was not complete (101). The two compounds each can destroy rat litters *in utero*; adenine protects against the destructive effects of diazo-oxo-norleucine, but not of azaserine (102). When tested against *Endamoeba histolytica*, azaserine was amoebicidal, whereas the other was without activity (103).

Barker and co-workers (103a) have studied the effects of azaserine on *Scenedesmus* during photosynthesis. In this system, azaserine caused an increase in glutamine and the Krebs cycle acids and a depletion in amino acids; the rate of formation of sucrose was increased and the photosynthetic carbon cycle was unaffected. These results suggest that azaserine inhibits a number of transaminations (103a).

In a study with purine-requiring mutants of *E. coli*, Gots & Gollub (104) observed that azaserine inhibited the synthesis of the aminoimidazole precursors of purines, which normally are accumulated by these mutants, and that inhibition could be reversed by aromatic amino acids and to a lesser degree by several other amino acids including  $\beta$ -2-thienylalanine. Since this last compound is an antagonist of phenylalanine, the suggestion was made that reversal of azaserine inhibition by aromatic amino acids results from a nonspecific chemical reaction rather than restoration of a metabolic block.

The evidence available at present indicates rather clearly that azaserine and diazo-oxo-norleucine may inhibit at multiple sites. Since these agents have been found to act as glutamine antagonists in several isolated systems and since glutamine has many known functions and doubtless many unknown, one might speculate that apparently conflicting results obtained in different biological systems eventually can be rationalized as glutamine antagonism. Differences in results of reversal studies in different biological systems might be postulated to result from variations in the relative importance to the system of a specific glutamine-requiring reaction or in the capacity of a given enzyme to bind glutamine or the inhibitor.

**6-Mercaptopurine.**—The profound effects of 6-mercaptopurine on a number of animal tumors and on leukemia in children (105) and the fact that it is the only purine analogue in widespread clinical use continue to

stimulate extensive interest in its mechanism of action. Structurally, it is an analogue of hypoxanthine or of adenine and evidence has for some time been available, in both microbiological and mammalian systems, which indicates that one mechanism of action is by antagonism of hypoxanthine derivatives, probably at the inosinic acid stage of purine synthesis (105, 106) (Figure 1). Recent work has added to the knowledge of the effects of 6-mercaptopurine on purine synthesis and has produced data indicating a mechanism more complex than that shown in Figure 1. Greenlees & LePage (98) found that the substance inhibited utilization of glycine for purine synthesis in ascites cells *in vitro*, in accord with earlier observations that, *in vivo*, it inhibited purine synthesis but did not inhibit the incorporation of preformed purines with the exception of hypoxanthine (105, 107). In human leukemic cells, *in vitro*, 6-mercaptopurine did not affect the conversion of adenine to guanine (108). In a rat liver extract, it was shown that the substance did not inhibit the incorporation of 4-amino-5-imidazolecarboxamide into purines (83) as would be expected if the incorporation were via the ribotide of 5-amino-4-imidazolecarboxamide (Figure 1); however, since the carboxamide occurs along this pathway as a nucleotide and not as the free base, it is possible that when the free base is administered it may be converted directly to adenine or guanine derivatives without passing through the ribotide and inosinic acid. Gots & Gollub (109) found that in a purine-requiring mutant of *E. coli* which accumulates 4-amino-5-imidazolecarboxamide the synthesis of the carboxamide could be inhibited by 6-mercaptopurine; since similar inhibition could be obtained by normal purines or by other purine analogues, it was postulated that in this instance the mercaptopurine was not inhibitory by a metabolic blockade in the usual sense but rather that, because of its structural similarity to natural purines, the inhibition was the result of a feedback mechanism by which the presence of the products of a biosynthetic reaction or their analogues inhibit synthesis of the metabolites.

Many lines of evidence implicate 6-mercaptopurine in metabolic areas other than purine metabolism. Since many coenzymes contain purines, any purine analogue may inhibit by antagonism of coenzyme nucleotides as well as by interference with nucleic acid metabolism. Garattini and co-workers have emphasized the role of 6-mercaptopurine as an antagonist of coenzyme A. *In vitro* and *in vivo*, it was found to inhibit acetylation of sulfanilamide (110, 111) and it also decreased cholesterol formation in regenerating liver (112). Other studies which implicate coenzyme A as a site of action are the observation of reversal by coenzyme A of the inhibition of mitosis and of lipogenesis induced in tissue culture systems by 6-mercaptopurine (113). Bolton & Mandel (114) found that in *E. coli* 6-mercaptopurine inhibited the utilization of acetate for synthesis of proteins and lipides and the incorporation of a number of precursors into nucleic acids; there was no effect on synthesis of proteins. The effects on nucleic acid synthesis in *E. coli* were analogous to those found in the mouse (114a). In mice, 6-mercaptopurine has been found to inhibit the synthesis and breakdown of diphosphopyridine nucleotide (115).

These results from a number of systems indicate that 6-mercaptapurine interferes with the functioning of purine nucleotides and may block metabolism at more than one point. There is no indication in any of the reported studies as to which site may represent the primary block; in this regard, however, it is interesting to note that in *E. coli* growth inhibition was significant before effects on acetate utilization or nucleic acid synthesis were detectable by tracer methods (114). 6-Mercaptapurine has been found also to affect respiration and glycolysis (116, 117, 118); respiration and anaerobic glycolysis were more sensitive to impairment in Sarcoma 180 than in liver or kidney (116).

The metabolic fate of 6-mercaptapurine has continued to attract attention. Balis & Hutchison (119) observed that  $C^{14}$  from labeled mercaptapurine appeared in the nucleic acids of *S. faecalis*; this was probably the result of conversion to hypoxanthine which is well utilized by this microorganism. The urine of humans who received 6-mercaptapurine- $S^{35}$  contained a  $S^{35}$ -labeled compound of a structure as yet undetermined (120).

Structure-activity relationships may often provide leads as to mechanism and a number of derivatives of the mercaptapurine have been prepared for this purpose (121). Thioguanine, the 2-amino derivative of 6-mercaptapurine, had higher toxicity than the latter, but about the same chemotherapeutic index. Substitution on the sulfur atom did not destroy activity, but replacement of the mercapto group with alkyl, halogen, cyano, or carboxy groups resulted in loss of activity or in inactive compounds (121). At equimolar levels, the riboside of 6-mercaptapurine showed the same antitumor activity as did the mercaptapurine, and lines of bacteria and tumors resistant to 6-mercaptapurine were cross-resistant to the riboside (122).

**8-Azaguanine.**—8-Azaguanine has been extensively studied for a number of years but there is as yet no clear-cut delineation of its mode of action in tumors. It is extensively incorporated into RNA of microorganisms and has been postulated to inhibit by formation of a fraudulent nucleic acid (123). It is also incorporated into nucleic acids of mammalian systems, though the incorporation is much smaller than in microorganisms and there is no compelling evidence that this is the mechanism of inhibition of tumor growth. Like 6-mercaptapurine, 8-azaguanine may be postulated to act at the nucleotide level, though again direct evidence is lacking.

Recent work on azaguanine has been largely in microbial systems. *Bacillus cereus* was found to incorporate azaguanine extensively into nucleic acids (123a to 126), and growth inhibition and incorporation into RNA were qualitatively related (126). Similarly, earlier results had shown a correlation between the incorporation of azaguanine into RNA and the infectivity of tobacco mosaic virus (123). Thus, in microorganisms there is good evidence that the incorporation of this purine analogue into RNA is the cause of, or is closely associated with, its ability to inhibit growth.

Some 8-azapurines are interconverted in much the same way as natural purines, as evidenced by the fact that in microbial systems 8-azaadenine, 8-azahypoxanthine, 8-azaxanthine, and 4-amino-1,2,3-triazole-5-carboxamide



are each incorporated into RNA as 8-azaguanine (126). In regard to utilization of 8-azaguanine by mammals, it is of interest that 8-azaguanine can be converted to its 5'-ribotide by incubation with 5-phosphoribosyl pyrophosphate in the presence of a liver enzyme system (127).

In mammalian systems *in vitro*, 8-azaguanine has been found to be an inhibitor of xanthine oxidase (128) and adenosine deaminase (129) but evidence is lacking that these observations are related to inhibition of tumor growth by 8-azaguanine *in vivo*.

*Other purine antagonists.*—The metabolism of purine and purine riboside has been studied by Gordon *et al.* (130, 131) who found that both compounds were extensively catabolized and that the riboside was used to a greater extent for synthesis of nucleic acid adenine and guanine than was the free base. The metabolism of 2,6-diaminopurine, another purine which inhibits growth of some tumors and is at the same time a precursor of nucleic acids, was studied by Remy (132) who found that *E. coli* converted it to 2-methylamino-6-aminopurine and the 5'-nucleotides of 2,6-diaminopurine and 2-methylamino-6-aminopurine. These observations on the metabolism of these compounds provide no obvious leads as to their mechanism of action.

The observation of the antitumor activity *in vivo* (133) and in tissue culture (134) of 4-aminopyrazolo(3,4-d)pyrimidine (APP) has stimulated considerable interest in this class of compounds. Of the large number of members of this series which have been tested *in vivo*, activity was found only in the 4-amino-, the 4-alkylamino-, and the 4-dialkylamino-compounds and their 1-alkyl derivatives (133, 135). Only borderline activity was obtained in the closely related series of pyrazolo(4,3-d)pyrimidines which were tested (135). Since APP is an isomer of adenine, it is attractive to postulate that the active members of this series act as purine antagonists. Inhibition of *Lactobacillus arabinosus* (135) and of *Neurospora crassa* (136, 137) by APP could be prevented by adenine; the significance of the observations in the latter system is clouded, however, by the fact that other pyrazolopyrimidines relieved inhibition by APP more effectively than did adenine (137). That APP may act by a mechanism independent of that of other purine antagonists such as 6-mercaptopurine, 2,6-diaminopurine, and 8-azaguanine is indicated by the fact that lines of *L. arabinosus* resistant to these agents as well as a neoplasm resistant to 6-mercaptopurine were still inhibited by APP (135). Certain pyrazolopyrimidines have been found to act as inhibitors of, and substrates for, xanthine oxidase *in vitro* (138); similar observations have been made with a number of other purine analogues and derivatives (138, 139). The pyrazolopyrimidines are thus different in this regard from 8-azaguanine which is a potent inhibitor of xanthine oxidase *in vitro* but cannot serve as a substrate for this enzyme (128, 138).

*Pyrimidine antagonists.*—The most interesting recent development in this area has been the preparation and biological study (140, 141, 142) of the 5-fluoro derivatives of pyrimidines and their nucleosides. 5-Fluorouracil and

5-fluoroorotic acid were found to be potent inhibitors of growth of a number of transplantable animal tumors, and the former has been evaluated in the clinic (141). These agents have been found to interfere with nucleic acid metabolism. In *Lactobacillus leichmanii*, the inhibitory activity of 5-fluorouracil was reversed by thymidine, thymine, 5-methylcytosine, uracil, and cytosine, whereas in *E. coli*, inhibition was completely reversed by uracil but was unaffected by thymine or thymidine, suggesting that different pathways were blocked in the two organisms (141). 5-Fluorouracil inhibited incorporation of uracil into RNA more effectively than did 5-fluoroorotic acid in glycolyzing suspensions of Ehrlich ascites cells. 5-Fluorouracil, its riboside, and its deoxyriboside inhibited incorporation of formate into nucleic acid thymine; since the deoxyriboside was much more effective than the riboside or free base, it was postulated that the active inhibitor was 5-fluoro-2'-deoxyuridylic acid (142). In slices of human tumors, 5-fluorouracil inhibited markedly the conversion of orotic acid to thymine with a much smaller effect on its conversion to other pyrimidines and increased the activity in DNA thymine when thymidine- $C^{14}$  was the precursor (143); in this same system 5-bromodeoxyuridine was highly effective in inhibiting incorporation of orotic acid into nucleic acid thymine, whereas 5-bromouridine and several related nucleosides were ineffective (144). These results are further evidence that 5-fluorouracil specifically blocks the methylation of the thymine precursor. The fluorouracil was incorporated into nucleic acids as such (141), but, as in the case of purine analogues which are incorporated, there is no evidence to indicate incorporation as the cause of growth inhibition.

The groups at Yale and at Prague have continued studies on the mechanism of action of 6-azauracil (145 to 152a). *E. coli* and *S. faecalis*, grown in the presence of azauracil, were found to accumulate the riboside (6-azauridine) in the medium or in the acid-soluble fraction of the cells (147, 151, 152a). 6-Azauracil inhibited the growth of Sarcoma 180 *in vivo*; in tissue culture, Sarcoma 180 was inhibited by azauridine, but not by azauracil (145, 148), and a strain of *S. faecalis* resistant to azauracil was strongly inhibited by azauridine (149). The results would suggest that azauracil, in order to be inhibitory, must be activated by conversion to its riboside or some other derivative (148, 149). The difference between the results with azauracil and azauridine and 6-mercaptopurine and its riboside (see above) should be noted.

Closely related to the investigations of azauracil and azauridine are those carried out with azathymine, azathymidine, and azathymidylic acid (153, 154, 155). Toward microorganisms azathymidine was more inhibitory than azathymine, suggesting, as with azauridine, that the active form is a nucleoside or a derivative thereof (154). Azathymine inhibited incorporation of formate into DNA of mammalian tissue *in vitro* (153) and was itself incorporated into DNA of *S. faecalis* (154). In studies of effects on the incorporation of thymidine into DNA *in vitro*, it was observed that azathymine did not inhibit and that azathymidine was a more effective inhibitor than the 5'- or 3'-phosphate or the 3',5'-diphosphate derivatives (155). This does

not exclude azathymidylic acid as the active inhibitor since there is evidence that nucleotides are dephosphorylated in passing through cell membranes.

6-Uracilsulfonic acid, 6-uracilsulfonamide, and 6-uracil methyl sulfone, which are structural analogues of orotic acid, were found to inhibit competitively the conversion of orotic acid to orotidine-5'-phosphate and to have no effect on the conversion of orotidine-5'-phosphate to uridine-5'-phosphate (156).

Guthrie and co-workers have continued study of analogues of the pyrimidine moiety of thiamine. Inhibition of bacteria by 2-methylmercapto-4-amino-5-hydroxymethylpyrimidine, shown earlier to have carcinostatic activity (157), could be reversed by thiamine or the pyrimidine moiety of thiamine; in animals, pyridoxine protected against the toxicity of the drug (158). Lines of bacteria resistant to amethopterin or 6-mercaptopurine showed enhanced sensitivity to this drug; it was postulated that the former prevented the synthesis of the pyrimidine moiety of thiamine whereas the pyrimidine antimetabolites interfered with its utilization (159, 160).

*Urethan and formamides.*—From the standpoint of mechanism of action, urethan is of interest as being one of the anticancer agents which has been longest known and which, along with formamide, has the simplest chemical structure. Despite much early work, its mode of action has remained unknown and interest therein has been largely displaced by study of other agents of wider clinical usefulness. Recently, Rogers (161), in a study of the carcinogenic action of urethan, has uncovered interesting new leads as to its mechanism.

Rogers observed that aminopterin, a known inhibitor of nucleic acid synthesis, potentiated the carcinogenic activity of urethan, and further, that the carcinogenic activity of urethan could be reversed by administration of pyrimidines and their derivatives or precursors: ureidosuccinic acid, dihydroorotic acid, orotic acid, cytidylic acid, or thymine. On the basis of these results, it was postulated that the action of urethan might result from its structural similarity to ureidosuccinic acid and it thus might interfere with the formation or utilization of carbamyl groups. It is attractive to speculate that, because of structural similarity to urethan, formamide and N-methylformamide, the latter of which has been shown to inhibit utilization of formate for purine synthesis (162), may have a similar mechanism. Evidence that such may be the case is the observation that lines of *E. coli* resistant to N-methylformamide or formamide were cross resistant to urethan (163).

*Other agents.*—The mechanism of action of alkylating agents has continued to attract widespread interest, but in spite of the many papers which have appeared during the year on the distribution and excretion of alkylating agents in animals (164 to 171) and their reaction with various cell constituents such as proteins, nucleotides, and nucleic acids (172, 173, 174), there is as yet no convincing evidence of their mode of action *in vivo*. Alkylating agents were the subject of a symposium held during the year, the papers

to be published shortly by the New York Academy of Science; no attempt will be made to summarize this material here.

Scattered studies have been reported on the mechanism of action of a number of other compounds of interest as carcinostatic agents. Several workers have extended observations on the anticancer activity of the actinomycins (175, 176, 177); studies in microbial systems (177, 178, 179) have indicated a pantothenate relationship, but mechanism studies have not been reported in mammalian systems. Bahner (180) has continued studies with 4-(*p*-dimethylaminostyryl)quinoline and related compounds. This agent is of interest in that it is effective only when administered orally, but no studies on its mode of action have been reported. The niacin antagonist, 6-amino-nicotinamide, has been found to be inhibitory towards several tumors (181, 182); the effects against Adenocarcinoma 755 could be reversed by simultaneous administration of nicotinamide (182).

Interest in the action of enzymes on tumor growth *in vivo* has continued. Bergel and his associates (66) have reviewed their recent work with xanthine oxidase and have indicated that a full scale biological trial is underway to evaluate the finding of Haddow that parenteral injection of xanthine oxidase preparations inhibited the growth of spontaneous mouse tumors. Following the observation of Ledoux (183) that ribonuclease had antitumor activity, further studies of this enzyme have been carried out, chiefly by Ledoux and his associates (184 to 187). Ledoux has summarized the evidence that ribonuclease can pass through cell walls and has obtained results which show that this enzyme causes disturbances in the metabolism of both RNA and DNA; growth inhibitory effects were ascribed to this imbalance in nucleic acid synthesis (186). These observations have an obvious relationship to the work of Cohen (52, 79) on imbalanced RNA and DNA synthesis as a cause of cell death in bacteria.

Significant antitumor activity has been reported in several new classes of compounds which will undoubtedly be the subject of future studies on mode of action. In mice, reserpine was found to have unequivocal antileukemic activity; whether this effect is mediated through the host has not yet been determined (188). Schneyer (189) found pilocarpine to have significant activity against two mouse adenocarcinomas and suggested that the effects might be ascribed to an enhanced immunological response of the host. Antitumor activity in one or more systems *in vivo* was also reported in studies with certain triazenes (190, 191),  $\beta$ -ethoxy- $\alpha$ -ketobutyraldehyde (192),  $\beta$ -dimethyl-aminoethylphenylpropyl acetate (193), dehydroascorbic acid (194), and certain 1,3-derivatives of benzoxazine (195), but studies of the mode of action of these agents have not been carried out.

Several observations have been made of the activity of agents against tumor cells in tissue culture, but the significance of these as chemotherapeutic agents must await trial in animals. 6-Furfurylamino-9- $\beta$ -D-ribofuranosyl-purine, the riboside of the plant-growth stimulator, kinetin, was found to be very toxic to fibroblasts as compared with three strains of human carcinoma

cells (196). Of interest is the report that the riboside of 2-fluoroadenine is more active against human tumor cells than against monkey kidney cells (197). 2-Azaadenine has been reported to be 10,000 to 100,000 times more toxic than 6-mercaptopurine to human carcinoma cells (198).

#### MECHANISMS OF RESISTANCE TO ANTICANCER AGENTS

The development of drug-resistance in populations of leukemic cells during the course of treatment looms as perhaps the most obvious stumbling block to achieving cures of this class of neoplastic disease and the existence of a "natural" refractory state (in most solid tumor cells in man) to inhibition by antileukemic agents prevents more widespread usefulness of these agents. Despite this, relatively little effort has been or is now being directed toward uncovering the underlying reasons for drug resistance in cancer cells.

On the surface, the problem of drug resistance in cancer cells might appear less complicated than uncovering the biochemical alterations responsible for cancer, because the experimental system may be limited to sensitive and resistant pairs of cells growing at similar rates *in vivo* or *in vitro*.

In spite of the importance of the problem of "acquired" and "natural" resistance to drugs in the treatment of cancer, less than twenty papers clearly oriented toward this subject have appeared in the literature during the past year.

An excellent review of the status of knowledge on drug resistance and cancer chemotherapy has recently been provided by Law (199). Therefore, only recent publications on this subject will be referred to here.

Hutchison & Coultas (200) have isolated amethopterin-resistant mutants of *S. faecalis* from cultures on purine-free and pyrimidine-free media and have noted significant differences in growth requirements of the resistant mutants obtained. The mutant isolated from cultures growing on a purine-free medium grew well on a folic acid medium devoid of purines and pyrimidines, whereas the mutant isolated from cultures on a purine-free, pyrimidine-supplemented medium did not grow in the absence of thymine, thymidine, or thymine riboside. Such results give support to the hypothesis that "in nature myriad pathways exist for successfully circumventing the effects of antimetabolites" (200).

Wacker *et al.* (201) in work with sulfanilamide-sensitive and resistant bacteria have observed that sulfanilamide-S<sup>38</sup> is taken up more extensively by the sulfa-resistant bacteria but that the drug-binding was weaker in the resistant organism. In the sensitive cells, sulfathiazole completely replaced *p*-aminobenzoic acid, but this did not take place in the resistant line. In the opinion of the reviewers, similar experiments on drug-binding and altered enzyme affinity should be carried out with anticancer agents. Although altered cell permeability is a resistance mechanism which apparently appeals to many investigators, resistance to a given drug should not be attributed to changes in cell permeability unless there are available experimental results that point to this mechanism.

Tomisek carried out studies *in vivo* with C<sup>14</sup>-formate in animals bearing

L1210 and L1210-amethopterin-dependent leukemias; a chromatographic-autoradiographic examination was made of the soluble labeled metabolites. It was noted that (56)

radioactivities of the observed metabolic pools indicated that the sequence of biochemical events from formate to various purine nucleotides were the same in the parent L1210 leukemia and the amethopterin-dependent leukemia.

Drew (202) has reported acquisition of resistance by a susceptible strain of *Diplococcus pneumoniae* as a result of a short exposure to the DNA derived from a strain of *Pneumococcus* resistant to amethopterin. Such results suggest in a most direct fashion that amethopterin resistance in this system is gene-linked.

Guthrie *et al.* (159) have found that several analogues of the pyrimidine moiety of thiamine are more inhibitory to amethopterin-resistant mutants of *Bacillus subtilis* and to mutants of *E. coli* resistant to purine antagonists than are the parent strains of these resistant organisms. This "collateral" sensitivity is believed to be related to the fact that in *B. subtilis* growth inhibition by amethopterin is effectively blocked by thiamine, and it has been suggested that the compound prevents *de novo* synthesis of the pyrimidine moiety of thiamine (159). Collateral sensitivity has previously been observed in the response of 8-azaguanine- and 6-mercaptapurine-resistant lines of L1210 leukemia to amethopterin (203, 204). More effort might wisely be applied in the search for agents with unusual activity against cells resistant to anticancer agents, both from the obvious practical importance of such knowledge and the theoretical implications of such information.

During the past year Brockman has reported that while *S. faecalis* (SF/O) will convert labeled adenine, hypoxanthine, guanine, or xanthine to other natural purines and purine nucleotides, a 6-mercaptapurine-resistant line (SF/MP) fails to convert hypoxanthine or guanine to other purines or nucleotides (205). Also it was noted that 6-mercaptapurine inhibits conversion of hypoxanthine and guanine, but not adenine and xanthine, to ribotides. These results are in general agreement with those obtained by Hutchison (206) who found that SF/O will grow in the absence of folic acid if adenine, hypoxanthine, xanthine, or guanine are provided, but that SF/MP grows only on xanthine (not on adenine, hypoxanthine, or guanine) in a folic acid-free media. These and other results have led to the hypothesis that 6-mercaptapurine-resistance may be associated with an inability of cells to convert certain purines and purine antagonists to ribotides. It has been demonstrated that 6-mercaptapurine-resistant bacteria and neoplastic cells are cross-resistant to 6-mercaptapurine riboside (122). The critical experiment with reference to the hypothesis that failure of certain cells to convert 6-mercaptapurine to a fraudulent ribotide is associated with resistance will be the demonstration that 6-mercaptapurine-resistant cells or biochemical events in extracts of such cells are not cross-resistant to 6-mercaptapurine ribotide.

Shacter & Law have quite convincingly demonstrated that 8-azaguanine-



resistant and 8-azaguanine-dependent lines of mouse leukemic cells do not possess higher levels, than the susceptible line, of the deaminase which will convert 8-azaguanine into 8-azaxanthine, a compound with no antitumor activity (207).

With regard both to the mechanism of action of azapurines and the "natural" resistance of bacteria and bacteriophage, Smith & Matthews (126) report that "inhibition of bacterial growth or virus development and incorporation of the analogue into RNA as 8-azaguanine are in all cases correlatable." These authors feel that

inhibition by azapurines can be more satisfactorily explained by the failure of at least some of the RNA containing 8-azaguanine to function properly, than by a concept of competitive inhibition of enzymes metabolizing the corresponding natural purine.

However, the hypothesis that 8-azaguanine-resistant cells differ from susceptible cells in their capacity to convert this antagonist to the corresponding ribotide (as has been proposed in the case of 6-mercaptapurine resistance) is still tenable. Although it has been observed that 8-azaguanine-susceptible cells incorporate much higher levels of 8-azaguanic acid (208) into polynucleotides than do resistant cells, it is impossible to say with certainty whether high levels of 8-azaguanic acid act at the nucleotide or polynucleotide level.

Handschrumer (149) has recently added to the knowledge of resistance to a pyrimidine antagonist, 6-azauracil, which possesses antitumor activity. With *S. faecalis*, it was noted that azauridine is a more potent inhibitor than azauracil and that only uridine would nullify the inhibition of the former; either uracil or uridine would prevent the inhibition by azauracil. The finding that azauracil-resistant cells are not cross-resistant to azauridine and that azauracil-resistant cells lack the capacity to convert azauracil to azauridine is convincing evidence that lack of capacity to carry out this formation of the riboside may be associated with azauracil resistance. It appears that a definite difference exists in purine-antagonist-resistant cells since 6-mercaptapurine-resistant cells are cross-resistant to 6-mercaptapurine riboside (122); however, data at hand do suggest that purine-antagonist-resistant cells lack the capacity to convert the antagonists to the corresponding ribotides.

In studies on purine biosynthesis in azaserine-sensitive and azaserine-resistant lines of a plasma cell neoplasm, Anderson *et al.* (209) have observed that cell-free preparations are able to carry out the reaction: formylglycinamide ribotide  $\rightarrow$  5-aminoimidazole ribotide  $\rightarrow$  5-amino-4-imidazolecarboxamide ribotide. Azaserine and 6-diazo-5-oxo-L-norleucine inhibited the conversion of formylglycinamide ribotide to 5-amino-4-imidazolecarboxamide ribotide in cell-free extracts of both sensitive and resistant cell lines. However, with intact cells, azaserine inhibited the incorporation of glycine into purines of the sensitive line, but not the resistant line. It may be hoped that this difference, observed in cell-free preparations and intact cells, will not

be assumed to be caused by changes in cell permeability until more direct evidence is at hand.

Sartorelli & LePage (210) have observed an essentially similar inhibition of labeled glycine into purines of a sensitive and azaserine-resistant ascites carcinoma for periods up to three hours; from this time on there was an increasingly greater incorporation of glycine into cellular purines of the resistant line. These results were interpreted to suggest either a more rapid destruction of the drug or a regeneration of enzyme in the resistant line.

Potter & Law (211) have developed azaserine-, diazo-oxo-norleucine-, and N-methylformamide-resistant sublines of an ascitic plasma cell neoplasm 70429. Cross-resistance of azaserine-resistant sublines to diazo-oxo-norleucine and N-methylformamide, of the diazo-oxo-norleucine-resistant subline to azaserine and N-methylformamide, and of the N-methylformamide-resistant subline to azaserine and diazo-oxo-norleucine were observed without evidence of change in sensitivity to amethopterin or 6-mercaptopurine.

#### REVIEWS

A number of reviews have appeared during the year which are pertinent to the subject matter of this chapter. Much material relating to chemotherapy, antimetabolites, and drug-resistance is contained in the following: the *Henry Ford Hospital International Symposium on the Leukemias* (212), the *Proceedings of the Third National Cancer Conference* (213), the *Ciba Foundation Symposium on the Chemistry and Biology of the Purines* (66), and the *Tenth Annual Symposium on Fundamental Cancer Research* (214). Two symposia sponsored by the American Cancer Society, one on the biochemical characteristics of tumors (5) and one on the role of hormones in cancer (215) provide excellent critical appraisals of the status of knowledge in these fields. Much material of a review nature is contained in chapters by Domagk on experimental cancer research and by Butenandt and Dannenberg on the biochemistry of tumors which appeared in the *Handbuch der Allgemeinen Pathologie* (216). Among other pertinent reviews are those by Timmis (91) on antifolic acids and antipurines as chemotherapeutic agents; by Goldin & Mantel (217) on therapy with combinations of drugs; by Bane, Conrad & Tarnowski (218) on combination therapy with ionizing radiation; and several chapters in Volume IV of *Advances in Cancer Research* (219).

Finally, the status of cancer chemotherapy—the ability to control disseminated neoplasia in man—will probably improve slowly even if the biochemist fails to shed more light on the basic problems mentioned in this review. The practical value of clear-cut knowledge of the biochemical lesions responsible for cancer cannot be forecast or assessed, since the acquisition of such fundamental knowledge has not proceeded far nor has it served as a guide to synthesis or selection of the array of chemicals which now make possible the cure of so many disseminated microbial diseases. However, it is difficult to conceive that accurate information on the basic biochemical differences between cancer cells and their normal cells of origin would not con-

tribute to a more efficient and productive direction of all cancer research and to more rapid progress toward the goal of such research.

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# CHEMISTRY AND BIOCHEMISTRY OF ANTIBIOTICS<sup>1</sup>

By E. B. CHAIN

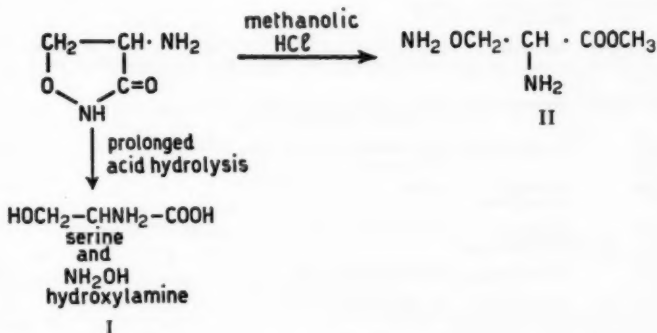
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From the list in Table I it is apparent that the search for new antibiotics is still continuing with vigour and is meeting with success. The field of antibiotics has become one of the major regions of natural compounds; the elucidation of their chemical structure, attempts at their partial or total synthesis, and the elucidation of their biosynthesis and their mode of action constitute a vast complex of problems of absorbing interest. This review is limited to progress in the chemical and biochemical aspects of the field following the chapter by Binkley (61) in 1955.

## ANTIBIOTICS OF AMINO ACID, PEPTIDE, AND POLYPEPTIDE NATURE

### AMINO ACID DERIVATIVES

*Cycloserine* (synonyms: *oxamycin* and *PA-94*).—A broad spectrum antibiotic with the elementary composition  $C_5H_8N_2O_2$  was isolated from culture filtrates of streptomycetes and studied in three different laboratories (24,



62, 63, 64). Its generally accepted name is now cycloserine. On mild hydrolysis it gave hydroxylamine and D-serine, on catalytic reduction D-serine amide, on treatment with methanolic hydrogen chloride  $\beta$ -aminoxy-D-alanine methyl ester (II)  $\text{NH}_2\text{OCH}_2 \cdot \text{CH} \cdot \text{NH}_2 \cdot \text{COOCH}_3$ . It follows from these facts that cycloserine possesses structure D-4-amino,3-isoxazolidone (I) (65). The compound has been synthesised (66, 67). Cycloserine shows therapeutic ef-

<sup>1</sup> The survey of the literature pertaining to this review was completed in September, 1957.

CHAIN  
TABLE I  
NEW ANTIBIOTICS

Antibiotic	Produced by	Active against	Chemical characteristics	Ref.
Actidiums	<i>Streptomyces</i> sp.	<i>Staphylococcus aureus</i> and other Gram-positive organisms	No definite melting point; cryst. as reineckate.	(1)
Aklavin	<i>Streptomyces</i> (A1163)	Bacterial phages	$C_{10}H_{12}NO_{11}$ (possibly closely related chemical individuals); cryst. as picrate, m.p. 168°; helianthate, m.p. 197°C.	(2)
Alazopeptine	<i>Streptomyces griseoplanus</i>	Gram-positive and Gram-negative bacteria; inhibits tumors	$C_{10}H_{12}N_7O_8$ ; no melting point.	(3)
Amphotericin A	<i>Streptomyces</i> (M 4575)	Fungi	No melting point, decomp. above 153°—Similar to the heptaenes.	(4)
Amphotericin B	<i>Streptomyces</i> (M 4575)	Fungi	$C_{10}H_{12}NO_{10}$ . No melting point, decomp. above 170°. Similar to the heptaenes.	(4)
Angolamycin	<i>Streptomyces eurythermus</i>	Gram-positive bacteria and protozoa	$C_{37.1}H_{59.1}NO_{11}$ ; m.p. 134–36° from benzol-ether, 165–68° from diisopropyl-ether. On hydrolysis gives two carbohydrates	(5)
Antibiotic 757	<i>Streptomyces</i> (757)	Antimitotic	Structurally closely related to ascocin	(6)
Antibiotic 899	<i>Streptomyces</i> sp.	<i>Streptococcus hemolyticus</i> in experimental infections	Structurally closely related to Streptogramin	(7)
Antibiotic 1703–18B	<i>Streptomyces</i> sp.		Structurally related to Hygromycin	(8)
Antibiotic 1968	<i>Streptomyces amidophilus</i>	Fungi		(9)
Antibiotic T-1384 (identical with Netropsin)	<i>Actinomyces</i>	Gram-positive and Gram-negative bacteria, and fungi	$C_{20}H_{24}N_{10}O_7$ —cryst. as picrate, m.p. 232°; helianthate, m. p. 215°.	(10)
Antibiotic X-465	<i>Streptomyces</i> sp.	Many actinomycetes, certain bacteria and bacterial phages	$C_{20}H_{24}O_{11}$	(11)
Antibiotic Substance	<i>Bacillus pumilus</i>	Gram-positive bacteria; non toxic	$C_8H_8N_2O_5S$ ; m.p. 252°	(12)
Antivirubin	<i>Actinomyces longispororuber</i>	Viruses		(13)
Bryamycin	<i>Streptomyces hawaiiensis</i>	Gram-positive bacteria	m.p. 223–35° polypeptide	(14)
BU 271	<i>Actinomyces</i> sp.	Gram-positive bacteria		(15)
BU 306	<i>Actinomyces</i> sp.	Gram-positive and Gram-negative bacteria		(16)
Campestrin	<i>Psalliota campestris</i>	Gram-positive and Gram-negative bacteria		(17)
Carsinophilin	<i>Streptomyces sakakirai</i>	Gram-negative bacteria and Yoshida sarcoma cells	Heat labile	(18)
Celesticetin	<i>Streptomyces coelestis</i>	Gram-positive organisms	$C_{20}H_{24}N_{10}O_7S$ —as oxalate m.p. 149°, as salicylate m.p. 139°	(19)
Cephalosporin C	<i>Cephalosporium</i>	Gram-positive and Gram-negative organisms; inhibits penicillinase	$C_{18}H_{21}N_7O_5S$	(20)
Cereine	<i>Bacillus cereus</i>	Shigella, Proteus, Escherichia, Salmonella, Serratia	Polypeptide	(21)

TABLE I—(Continued)

Antibiotic	Produced by	Active against	Chemical characteristics	Ref.
Chryomycin	<i>Streptomyces</i> (A-419)	Bacterial phages	$C_{11}H_{10}O_7$	(22)
Comirin	<i>Pseudomonas antismycelica</i>	Fungi	m.p. 230–35°, with decomposition	(23)
Cycloserine (synonymous with oxamycin, PA-94)	<i>Streptomyces orchidaceus</i> and other strains	Gram-positive and Gram-negative organisms	$C_8H_{12}N_2O_3$ m.p. 156°	(24)
DON	<i>Streptomyces</i> (P-D 04997)	A few strains of Gram-positive and Gram-negative bacteria and sarcoma 180 in mice	$C_8H_8NaO_3$ d.p. 145–55°	(25)
Echinomycin	<i>Streptomyces echinatus</i> n. sp.	Gram-positive bacteria	$C_{18}H_{18}N_7O_7S$ ; m.p. 217–18°	(26)
Etamycin (synonymous with Viridogrisein)	<i>Streptomyces</i> sp.	Gram-positive bacteria and Myco. tuberculosis	Polypeptide m.p. 163–70°	(27)
Eulicin	<i>Streptomyces</i> sp.	Fungi		(28)
Filipin	<i>Streptomyces filipinensis</i>	Fungi	$C_{40}H_{50}O_{10}$ ; 2 solid modifications, transition at 147°; m.p. of second form 195–205°	(29)
Flavensomycin	<i>Streptomyces lanaschiensis</i>	Fungi	152 ± 2°	(30)
Flavipin	<i>Aspergillus flaviceps</i>	Fungi	$C_8H_8O_3$ ; m.p. 233–34° with decomposition	(31)
Foromacidsins A, B, C, D. (Probably identical with spiromycins A, B, C.)	<i>Streptomyces</i> sp.	Gram-positive bacteria	$C_{10}H_{12}N_7O_{10}$ m.p. 134–38° $C_{10}H_{12}N_7O_{10}$ 130–32° $C_{10}H_{12}N_7O_{10}$ 124–28° 135–40°	(32)
Fungichromatin	<i>Streptomyces</i> sp.	Fungi		(33)
Fungichromin	<i>Streptomyces cellulosa</i>	Fungi	$C_{18}H_{18}O_{10}$ ; m.p. 205–10°	(33)
Griseoviridin	<i>Streptomyces griseus</i>	Gram-positive and Gram-negative bacteria	$C_{11}H_{14}N_4O_7S$ ; m.p. 228–30°	(34)
Miamycin	<i>Streptomyces ambofaciens</i>	<i>M. pyogenes</i> var. <i>aureus</i> made resistant to erythromycin and carbomycin	m.p. 221–22°	(35)
Mollisine	<i>Mollisia caesia</i> <i>Mollisia fallens</i>	Fungi		(36)
Mycolutein	<i>Streptomyces</i> sp.	Fungi	$C_{18}H_{18}NO_4$ ; m.p. 157–58°	(37)
Mycothricin	<i>Streptomyces lavendulae</i>	Gram-negative, Gram-positive bacteria and fungi	polypeptide	(38)
Narbomycin	<i>Streptomyces narbonensis</i>	Inactive <i>in vivo</i>	$C_{28}H_{40}O_7N$ ; m.p. 113.5–15°, macrolide	(39)
Neomethymycin			$C_{21}H_{24}NO_7$ ; m.p. 156–158°	(40)
Novobiocin (synonymous with Cardelmicin, Cathomycin, Streptonivicin, Albamycin, PA-93)	<i>Sirept. spheroides</i> <i>Sirept. niveus</i> <i>Sirept. griseus</i>	Gram-positive and a few Gram-negative bacteria	$C_{11}H_{18}N_4O_{11}$	(41)
Nucleocidin	<i>Streptomyces calvus</i>	Gram-positive and Gram-negative bacteria	$C_{11}H_{18}N_4O_8S$ ; m.p. as picrate 143–44°	(42)
Nybomycin	<i>Streptomyces</i> (A-717)	Bacterial phages	$C_8H_7NO_3$ ; s.p. 250°	(43)



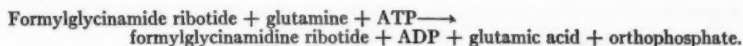
TABLE I—(Continued)

Antibiotic	Produced by	Active against	Chemical characteristics	Ref.
Oleandomycin (PA 105)	<i>Streptomyces antibioticus</i>	Gram-positive bacteria, mycobacteria, rickettsiae, large viruses and certain protozoa.	Macrolide m.p. 134–35°	(44)
PA 95	<i>Streptomyces rosachromogenus</i>	Human strain of <i>Mycobacterium tuberculosis</i>	$C_{28}H_{42}NO_8S$	(45)
PA 114 A PA 114 B	<i>Streptomyces olivaceus</i>	Exhibit synergism in combination toward Gram-positive bacteria	$C_{28}H_{42}N_2O_8$ or $C_{28}H_{42}N_2O_9$ $C_{28}H_{42}N_2O_{12}$	(46)
PA 132	<i>Streptomyces</i> sp.	Gram-negative and Gram-positive bacteria, fungi and protozoa	$C_{28}H_{42}NO_8$ ; m.p. 128–31° as benzylamine salt	(47)
Pleomycin	<i>Streptomyces pleofaciens</i>	Gram-positive and Gram-negative bacteria	$C_{28}H_{42}O_8$ ; m.p. 235° uncorrected	(48)
Pumilin (probably identical with micrococin)	<i>Bacillus pumilus</i>	Gram-positive bacteria; highly toxic	m.p. 360°	(49)
Ramnacin	<i>Streptomyces</i> sp.	Bacteria and fungi	$C_{28}H_{42}O_8$ ; m.p. 235°	(50)
Ristocetin A) Ristocetin B)	<i>Nocardia lurida</i>	Gram-positive bacteria and mycobacteria		(51)
Rubidin	<i>Streptomyces</i> sp.	Gram-positive bacteria	Quinoid	(52)
Spiramycin A,B,C, (probably identical with foromicidins A,B,C,D.)	<i>Streptomyces ambofaciens</i>	Gram-positive bacteria	Macrolide	(53)
Streptolydigin	<i>Streptomyces lydicus</i>	Gram-positive bacteria	$C_{28}H_{42}N_2O_8$ or $C_{28}H_{42}N_2O_{12}$ m.p. 144–50°	(54)
Streptovaricin	<i>Strept. spectabilis</i>	<i>Mycobacterium tuberculosis</i>	(consists at least of five microbiologically active components, A, B, C, D, E, differentiated by paper chromatography)	(55)
Thermoviridin	<i>Thermooctino-mycetes viridis</i>	Gram-positive bacteria		(56)
Thiostrepton	<i>Streptomyces</i> sp.	Gram-positive bacteria	Polypeptide	(57)
Valinomycin	<i>Streptomyces fulvissimus</i>	<i>Mycobacterium tuberculosis</i>	$C_{28}H_{42}N_2O_{12}$ ; m.p. 190°; macrolide	(58)
Vancomycin	<i>Streptomyces orientalis</i>	Gram-positive bacteria and spirochetes		(59)
Zaomycin	<i>Streptomyces</i> sp.	Gram-positive bacteria and nonpathogenic mycobacteria	m.p. 242–46°	(60)

fectiveness against tubercle infections *in vivo* though the tubercle bacillus appears to be relatively insensitive to its action *in vitro* (68).

**Azaserine.**—This antibiotic, O-diazoacetyl-L-serine (69), discovered through its tumour-inhibiting properties (70) was shown to inhibit *in vivo* the incorporation of  $^{14}C$ -formate, glycine-1- $^{14}C$  and  $\beta$ - $^{14}C$ -serine into tissue nucleic acids, and the incorporation of  $^{14}C$ -formate and  $^{14}C$ -glycine into tumour nucleic acids (71). Only the incorporation of radioactivity into the adenine and guanine moieties of the nucleic acids was reduced by azaserine indicating that it interfered specifically with purine synthesis. The incorporation of precursor purines into nucleic acid was not influenced by azaserine.

Inhibition of purine synthesis by azaserine in different tumours and spleen tissue was also noted by Le Page & Greenlees (72), Heidelberger & Keller (73) and Fernandes, Le Page & Lindner (74). Interference of azaserine with purine synthesis had also been demonstrated by Hartmann, Levenberg & Buchanan (75) who showed that the *de novo* synthesis of inosinic acid in pigeon liver extract was inhibited by this antibiotic with the accumulation of formyl glycinamide ribotide. This compound, as well as formyl glycinamide riboside, was also accumulated in the cells of partially azaserine inhibited *E. coli* cultures (76). In these bacteria too the incorporation of  $^{14}\text{C}$ -formate,  $^{14}\text{C}$ -glycine and  $\beta$ - $^{14}\text{C}$ -serine into purines was inhibited. The inhibitory effect of azaserine on purine synthesis, both in the pigeon liver system (75) and *E. coli* (71) was abolished by glutamine. Tomisek *et al.* (76) concluded that azaserine interfered with purine synthesis at a stage subsequent to the formation of formyl glycinamide ribotide, possibly as competitive glutamine antagonist. Further information concerning the mode of action of azaserine and evidence that it acts as a competitive inhibitor to glutamine was obtained by Levenberg, Melnick & Buchanan (77) who showed that of the various stages in the synthesis of inosinic acid it inhibited specifically the reaction:



The growth inhibition of *E. coli* by azaserine was overcome by phenylalanine, tyrosine and tryptophan (71, 78), methionine, various purines and 4-amino, 5-imidazole carboxamide (71) indicating that azaserine had different sites of action in the bacterial metabolism.

**DON and alazopeptine.**—Two antibiotics related to azaserine have been isolated from streptomycetes. One of these had the elementary composition  $\text{C}_6\text{H}_9\text{N}_3\text{O}_5$ , liberated nitrogen on acidification and was shown to be 6-diazo, 5-oxo-L-norleucine, abbreviated DON (25). The compound was synthesised (79). Like azaserine, it showed limited activity against tumours (80, 81) and inhibited the incorporation of  $^{14}\text{C}$ -labelled formate and glycine into nucleic acids (82, 83). The second, termed alazopeptine, had the composition  $\text{C}_{18}\text{H}_{21}\text{N}_7\text{O}_6 \cdot \text{H}_2\text{O}$ , and was a peptide composed of one molecule of  $\alpha$ -alanine and two molecules of 6-diazo-5-oxo-amino hexanoic acid (DON or an isomer) (3). DON inhibited the same reaction as azaserine (see above), but in lower concentrations, and the inhibition was abolished by glutamine (77).

#### ANTIBIOTICS OF PEPTIDE NATURE

**Penicillin.**—The synthetic studies of Sheehan and his group on substances related to penicillin have been continued. By reacting *dl*-thiovaline (penicillamine) with *t*-butyl phthalimido malonaldehyde in aqueous alcohol buffered with sodium acetate the thiazolidine, *t*-butyl 4-carboxy-5, 5-dimethyl- $\alpha$ -phthalimido-2-thiazolidine-acetate was obtained (84) which, on treatment with diazomethane, yielded two of the four theoretically possible racemic stereoisomeric methyl esters. The lower melting racemate, m.p. 121–122°,

was the main reaction product; it was the  $\gamma$ -form usually predominant in synthetic penicilloates. The higher melting racemate, m.p. 176–176.5°, corresponded to the  $\alpha$ -form of the natural penicilloates. It was obtained in low yields, but further amounts could be obtained from the  $\gamma$ -form by refluxing in triethylamine which gave an equilibrium mixture of  $\alpha$ - and  $\gamma$ -form in the ratio 2:5.

By removing the phthalyl group with hydrazine the intermediate *t*-butyl, 4-carbomethoxy-5, 5 dimethyl,  $\alpha$ -amino 2 thiazolidine acetate (III)<sup>2</sup> was obtained in good yield.

This intermediate is of special interest because it affords an easy method of synthesis of different penicilloates by simple acylation for which the only hitherto available route was the condensation of  $\beta$ -thiolvaline and the sometimes difficultly accessible penaldates.

From the  $\alpha$ - and  $\gamma$ -isomers the *t*-butyl group was removed by treatment with anhydrous HCl at 0°C. in nitromethane and the corresponding free acids, the  $\alpha$ - and  $\gamma$ -isomers of 4-carbomethoxy, 5,5-dimethyl  $\alpha$ -phthalimido-2-thiazolidine acetic acid (IV), were obtained in good yield in form of the hydrochlorides. At higher temperatures, from 30° to 90°, isomerisation to another isomer, designated as  $\beta$ -form, took place, while at still higher temperatures there was decarboxylation to 2-phthalimidomethyl, 4-carbomethoxy, 5,5-dimethyl thiazolidine.

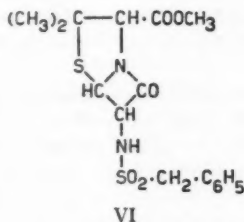
The  $\alpha$ - and  $\gamma$ -isomers of IV were converted into the corresponding dimethyl penicilloates by treatment with diazomethane, removal of the phthalyl group with hydrazine and phenylacetylation of the resulting  $\alpha$ -amino acids. The infrared spectrum of the  $\alpha$ -form proved identical in all respects with natural D- $\alpha$ -penicilloate.

Many attempts have been made in the past to cyclise penicilloates to  $\beta$ -lactams, but have met with no success, because, as might be expected, owing to the presence of the enolisable peptide side chain, azlactonisation occurred in preference to the formation of the  $\beta$ -lactam ring and simultaneous splitting of the thiazolidine ring led to the formation of penicillanates. In the phthalimido derivative no enolisation of the side chain is possible; hence fresh attempts to cyclise this substance were made with the  $\alpha$ -,  $\beta$ - and  $\gamma$ -isomers, using a number of cyclising agents under different conditions (85). Cyclisation to the  $\beta$ -lactam did take place with SOCl<sub>2</sub> and POCl<sub>3</sub> as cyclising agent, but only with the  $\beta$ -isomer; in the  $\alpha$ - and  $\gamma$ -forms  $\beta$ -lactam formation did not occur, probably due to steric hindrance. Methyl 6-phthalimidopenicillanate [for nomenclature see Sheehan, Henery-Logan & Johnson (86)] was isolated in crystalline state, m.p. 173–173.5°, in yields of 20 to 30 per cent. Reaction of this substance with hydrazine did not afford the desired 6-aminopenicillanate, but led to simultaneous splitting of the fused thiazolidine- $\beta$  lactam ring with the formation of the hydrazide. Methyl 6-phthalimidopenicillanate could be converted to the sulphone IV, m.p. 200–201° decomp.

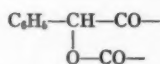
<sup>2</sup> Some compounds have been numbered to facilitate reference in the text. Their structures may readily be deduced.

In order to avoid the steric hindrance difficulties encountered in attempts to cyclise the  $\alpha$ - and  $\gamma$ -isomers of the phthalimido derivative the analogous benzylsulphonamide derivatives (V) which, like the former, do not give rise to azlactones on treatment with cyclising agents, were synthesised (87) by treatment with benzylsulphonyl chloride of the  $\alpha$ - and  $\gamma$ -isomers of intermediate III.

Removal of the *t*-butyl group by anhydrous HCl in nitromethane from the  $\alpha$ - and  $\gamma$ -isomers of V led to the isolation of the  $\alpha$ - and  $\beta$ -isomers of the free acid (Va), the  $\gamma$ -form of V undergoing isomerisation. Cyclisation of the  $\alpha$ - and  $\beta$ -forms of Va was achieved by refluxing in methylene chloride with thionyl chloride and both  $\alpha$ - and  $\beta$ -forms of methyl-6-benzyl-sulphonamido penicillinate (VI) were isolated in the crystalline state, m.p. of the  $\alpha$ -form 214–215°C., m.p. of the  $\beta$ -form 128–130°C. The  $\alpha$ -form possessed weak biological activity (7 units/mg) when tested *in vivo* in experimental infections in mice against *D. pneumoniae* Type II.



A synthesis of the sulphone of a racemate of benzyl penicillin methyl ester has been announced by Sheehan, but no experimental details have as yet been published (88). The route involves the synthesis of a penicilloate in which the protecting group



is introduced to prevent enolisation in the same way as was achieved by the phthalyl group. Ring closure followed by permanganate oxidation led to the sulphone of the  $\beta$ -lactam; on catalytic hydrogenation  $\text{CO}_2$  was eliminated from the protecting group with the concomitant formation of the benzyl side chain.

Recently, Sheehan & Henery-Logan (89) have reported the total synthesis of the acid stable phenoxymethylpenicillin. By condensing D-penicillamine with *t*-butyl-phthalimidomalonaldehyde the D- $\alpha$  form of phthalyl penicilloate was obtained which, on removing the phthalyl group with hydrazine, gave the D- $\alpha$  form of III. Acylation of this compound with phenoxylacetylchloride gave  $\alpha$ -*t*-butyl D- $\alpha$ -phenoxymethylpenicilloate. The free acid obtained by hydrolysis of the *t*-butyl ester by dry hydrogen chloride,

was converted to the potassium salt. Ring closure of the latter to the potassium salt of phenoxymethylpenicillin was brought about by cyclising with  $N,N'$ -dicyclohexyl-carbodiimide in dioxane-water. Aliphatic carbodiimides had previously been shown to form peptide bonds under very mild conditions from amine and carboxylic compounds (90).

Halliday & Arnstein (91) showed, in extension of previous work by Rolinson (92), that mycelium of *P. chrysogenum* grown on the synthetic medium of Jarvis & Johnson (93) for 68 to 70 hr., and transferred after filtering and washing to a lactose-corn steep medium, continued to produce penicillin for at least five hours at a uniform rate. This rate was maintained when either corn steep or lactose was omitted from the replacement medium, and it was reduced by about 20 per cent when both these constituents were omitted. Penicillin production continued at about 40 per cent the rate in the complete replacement medium in a solution containing nothing but 0.1 per cent potassium phenylacetate.

In the phenylacetate replacement medium  $L$ - $^{14}C$ -valine (uniformly labelled),  $L$ - $^{35}S$ -cystine and  $Na_2^{35}SO_4$  were incorporated into the penicillin molecule. Cyanide inhibited penicillin production to a larger extent than it inhibited respiration.

Brandl, Carilli & Chain (94) showed that reasonable yields of penicillin could be obtained in shake flasks on synthetic media containing one single amino acid as nitrogen source, lactose as carbon source and inorganic sulphate or methionine as sulphur source. Arginine gave the highest yields (1500 to 2000 units/ml. after 120 hr.), but good yields (over 1000 units after 120 hr.) were obtained with alanine, citrulline, glycine, leucine, ornithine, proline and tyrosine. The dibasic amino acids were poorly utilized, singly as well as in mixtures with other monobasic amino acids. In replacement experiments Brandl, Carilli & Chain (95) found that a 48-hour-old mycelium grown on corn steep-dextrin or corn steep-lactose, filtered and resuspended in different replacement media, such as corn steep-lactose, the synthetic medium of Jarvis & Johnson, and the arginine-sulphate or arginine-methionine medium, gave the same high penicillin titres at the same rapid rate as when grown in these media in the normal way. Washing of the seed mycelium did not affect titres and rate in the corn steep-lactose and Jarvis-Johnson replacement medium, but reduced it to less than half in the arginine replacement medium. The rate could be restored by adding small amounts of the seed culture filtrate, indicating the presence of some factor essential for penicillin production. Under the conditions of the replacement experiments new mycelial growth was very restricted. Penicillin production continued on a replacement medium containing only lactose as energy source and inorganic sulphate, and yields over 1000 units/ml. were obtained. Under these conditions the penicillin molecule was synthesised entirely from intracellular precursors in the absence of new mycelial growth. In the absence of the energy source no penicillin production took place and autolysis of the mycelium occurred. Arnstein & Grant (96), growing mycelium of *P. chrysogenum* on the

synthetic medium of Jarvis & Johnson to which ( $^{35}\text{S}$ — $^{14}\text{C}$  $\text{CH}_2$ — $\text{CH}^{15}\text{NH}_2$ — $\text{CO}_2\text{H}$ ) $_2$  was added, showed by stepwise degradation of the penicillin formed under these conditions that the cysteine molecule was incorporated intact into the penicillin molecule isotopic labelling appearing in the nitrogen of the side chain (position 4), the sulphur atom (position 1) and the adjacent CH of the thiazolidine ring (position 5). Arnstein & Clubb (97), furthermore, demonstrated that the total carbon chain of the valine molecule is incorporated intact into the penicillamine moiety of the penicillin molecule.

Stevens, Inamine & De Long (98) found that while  $^{14}\text{C}$ -labelled L-valine is incorporated into penicillin readily and immediately, the rate of incorporation of D-valine becomes rapid only after a lag period of 6 hr., during which time interval the rate of incorporation is very slow. According to the authors these findings indicate that D-valine may not be a direct precursor of penicillin; on the other hand, according to Arnstein & Clubb (97) differences in the rate of penetration of the two isomers into the cell may be responsible for the effect. To shed light on how the fused thiazolidine- $\beta$ -lactam ring is formed from cystine and valine, Arnstein & Crawhall (99) used  $\alpha$ - and  $\beta$ -tritium-labelled cystine as tracer, employing the replacement medium technique referred to above. The label from  $\alpha$ -labelled cystine appeared mainly in position 6, from  $\beta$ -labelled cystine in position 5, indicating the possibility of the formation of the  $\beta$ -lactam ring by oxidative condensation between the  $\beta$ -carbon of the cysteine moiety with the peptide nitrogen of a cysteinyl valine, simultaneously with the oxidative formation of the thiazolidine ring from the sulphur of the cysteine moiety and the  $\beta$ -carbon of the valine moiety. Ballio *et al.* (100), incubating washed mycelium of *P. chrysogenum* on the arginine replacement medium referred to above with radioactive sulphate, found radioactive sulphur in mycelial extract in the following compounds: inorganic sulphate as calcium salt, choline sulphate, methionine, cysteine, biotin, and an unidentified substance.

Park & Strominger (101) found that cell wall material from penicillin sensitive staphylococci yielded on acid hydrolysis D-glutamic acid, L-lysine, DL-alanine and 3-O-carboxy ethyl hexosamine [a sugar isolated by Cummins & Harris (102, 103)] from several species of Gram-positive bacteria whose structure was elucidated by Strange (104) in the proportions 1:1:3:1. Hydrolysis of the uridine nucleotide containing an acetyl hexosamine peptide moiety, which was previously shown by Park & Johnson (105) to accumulate in penicillin inhibited staphylococci, gave the same products in the same proportions. On the basis of these findings Park & Strominger suggest that the mechanism of the bactericidal action of penicillin may be an interference with the synthesis of bacterial cell wall material for which the uridine nucleotide is an intermediate.

*Cephalosporins.*—A review on these interesting antibiotics produced by a species of the filamentous fungus cephalosporium was published by Newton & Abraham (106). Seven different antibiotics were isolated. Of these, five, termed cephalosporin P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, P<sub>4</sub> and P<sub>5</sub>, appeared to be related in their

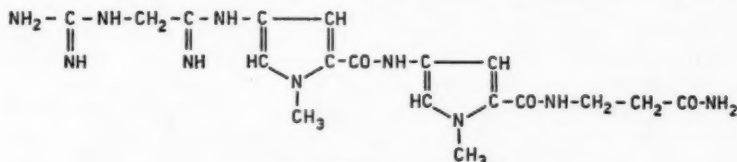




coccal penicillinase it has a strong synergistic effect on benzylpenicillin in its action against penicillin-resistant staphylococci (113). Both cephalosporins-N and -C are inducers of penicillinase formation in *B. cereus*, the latter being the most potent inducer known (114).

**Echinomycin.**—Echinomycin, an antibiotic highly active against Gram-positive bacteria, was isolated in crystalline forms from culture filtrates of a streptomycete. It has the elementary composition  $C_{29}H_{37}O_7N_7S$  (26). On acid hydrolysis (115) it yielded D-serine, L-alanine, N-methylvaline and on alkaline hydrolysis an acid  $C_9H_8O_2N_2$  which was identified as quinoxaline 2-carboxylic acid.

**Netropsin** (synonyms: *T-1384*, *sinanomycin*, *congocidin*).—An antibiotic with the elementary composition  $C_{18}H_{26}N_{10}O_3$  was isolated by independent groups of investigators from strains of streptomyces and was given the above-mentioned different names (10, 116, 117, 292). The elucidation of its structure was recently reported (10) as  $\beta$ [4-(4-guanidinoacetamidino-1-methyl 2-pyrrolicarboxamido)-1-methyl-2 pyrrolicarboxamido]-propionamide (VIII).



NETROPSIN

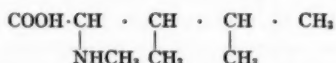
VIII

On mild alkaline hydrolysis it is hydrolysed into glycocyamidino and  $\beta$ [4-(4-amino, 1-methyl, 2-pyrrolicarboxamido) 1-methyl, 2-pyrrolicarboxamido] propionamide (IX). On further alkaline hydrolysis with 0.5N NaOH this component gave ammonia and the corresponding carboxylic acid (X) which, on hydrolysis with 5N NaOH, was hydrolysed to 2 moles of 4-amino-1-methyl-2 pyrrolicarboxylic acid (XI) and  $\beta$ -alanine. On treatment of VIII with  $\text{Ba}(\text{OH})_2$  one mole of ammonia is liberated and  $\beta$ -[4-(4-guanidinoacetamido-1-methyl-2 pyrrolicarboxamido)-1-methyl-2-pyrrolicarboxamido]-propionamide (XII) is formed. The breakdown products IX, X, XI and XII were synthesised (118).

**Micrococcin-P.**—A strain of *Bacillus pumilus* isolated from East African soil was found to produce an antibiotic active *in vitro* in high dilutions against *Pasteurella muriseptica*, *Streptococcus aureus* and *Streptococcus haemolyticus* and giving protection against the latter in experimental infections in mice (119). Investigations of its chemical and biological properties showed that it was similar to the previously described micrococcin (120, 121) and it was termed micrococcin-P (122). On acid hydrolysis this antibiotic yielded ammonia, L-threonine and two thiazole-4 carboxylic acids, 2-propionyl thiazole-4 carboxylic acid and 2(1-amino, 2-methyl propyl) thiazole-4-carboxylic acid

(123). The isolation of these thiazole 4-carboxylic acid derivatives is of considerable interest in connection with the reported isolation of 2 (1-methyl butyryl) thiazole-4-carboxylic acid from bacitracin-F (see below) (124). The thiazole derivatives isolated from micrococcin-P are considered to have derived from peptide chains formed of cysteine and adjacent amino acids with subsequent conversion to thiazolines and thence thiazoles. 2 Propionyl-thiazole-4-carboxylic acid would evidently derive from cysteine and  $\alpha$ -amino-butyric acid, 2(1-amino, 2 methyl propyl) thiazole-4-carboxylic acid from cysteine and valine.

*Griseoviridin and viridogrisein* (synonym: *etamycin*).—These antibiotics were isolated from culture filtrates of *Streptococcus griseus* and an unidentified *Streptococcus* sp. (34, 125). Griseoviridin, a colourless neutral compound, has the composition  $C_{22}H_{29}O_7N_4S$ , gives a diacetyl derivative, and the nitrogen appears to be present in amide linkages (126). On alkaline hydrolysis ammonia and sulphide were formed. On acid degradation of griseoviridin, cystine and serine were detected chromatographically. Acid degradation of octahydrodethiogriseoviridin diacetate, obtained from griseoviridin diacetate by desulphurisation with Raney nickel, gave 10-amino decanoic acid and two moles of D- $\alpha$ -alanine (127, 128). A partial structure, containing two amide linkages and one diacylamino group, is proposed for griseoviridin. Viridogrisein has the composition  $C_{44}H_{52}N_8O_{10}$ . On acid hydrolysis L- $\alpha$ -alanine, D-leucine and D-hydroxyproline, and 3-hydroxy picolinic acid were isolated (129). Chemical investigation of the antibiotic etamycin (27) showed it to be identical with viridogrisein. Sheehan, Zachau & Lawson (130) showed that, in addition to the above-mentioned amino acids, it contained sarcosine and two amino acids not previously encountered in natural products: L- $\beta$ ,N-dimethylleucine



and L-phenylsarcosine. They established the complete amino acid sequence in the etamycin molecule as 3-hydroxypicolinic acid-threo-D-leu-D-allohydroxypro-dimethyleu-L-ala-L-phenylsarc and demonstrated the presence of a macrocyclic lactone ring linking the hydroxyl group of threonine with the carboxyl of the terminal phenylsarcosine in a 22-membered ring.

#### ANTIBIOTICS OF POLYPEPTIDE NATURE

Craig has reviewed recent work on the structure of antibiotics of polypeptide nature to which he and his collaborators have made so many fundamental contributions. The results are of great general interest.

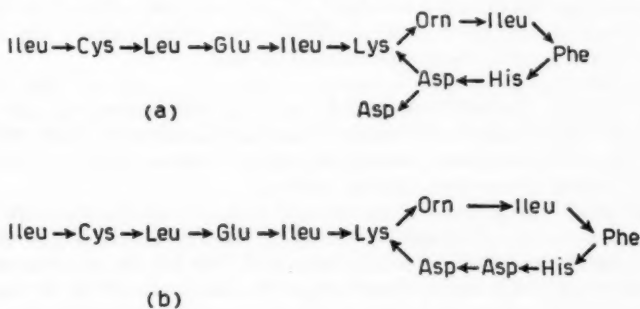
*Gramicidins*.—Gramicidin-S has been shown to be a cyclic decapeptide (Val.Orn.Leu.Phe.Pro)<sub>2</sub> (131) and this structure has been confirmed by Schmidt, Hodgkin & Oughton (132) by crystallographic x-ray analysis on a

number of salts as well as various acyl derivatives. It is assumed that the decapeptide has a twofold axis of symmetry. A number of molecular models for gramicidin-S have been put forward by Hodgkin & Oughton (133).

The straight chain decapeptide had previously been synthesised by Erlanger, Sachs & Brand (134).

The complete synthesis of the cyclic decapeptide has now been reported by Schwyzer & Sieber (135). It was achieved by cyclisation of the ditosyl decapeptide-*p*-nitrophenylester trifluoro acetate in dimethyl formamide at 55°C. and subsequent removal of the tosyl residues by reduction with sodium in liquid ammonia. The former was obtained from the trityl ditosyl decapeptide-*p*-nitrophenyl ester by removal of the trityl group with trifluoroacetic acid. The trityl-ditosyl-decapeptide *p*-nitrophenyl ester was obtained by treatment of the free acid with di (*p*-nitrophenyl)-sulphite in pyridine. The free trityl ditosyl decapeptide was obtained by alkaline hydrolysis of its methylester which was made by condensation of two moles of the trityltosyl-pentapeptide methylester, by means of the carbodiimide method. The trityl peptide was obtained by tritylation of L-valyl-N $\delta$ -tosyl-L-ornithyl-L-leucyl-D-phenylalanyl, L-proline methyl ester hydrochloride, a substance already synthesised by Erlanger *et al.* The synthetic product is identical with the natural antibiotic in melting point, optical rotation, infrared spectrum, x-ray analysis and antibacterial activity.

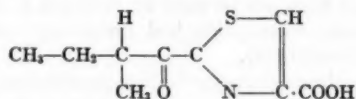
**Bacitracin.**—On the basis of studies on the structure of products of partial hydrolysis of bacitracin-A (136, 137) and trifluoro-dinitrophenyl bacitracin (138), two alternatives for the sequence of amino acids in this antibiotic have been suggested (XIIIa and b). The peptides appearing on partial hydrolysis of bacitracin-A were purified by countercurrent distribution.



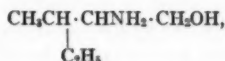
### XIII

Further evidence for the suggestion (139) that the cysteine moiety in bacitracin is present in form of a thiazoline ring linked to isoleucine has been obtained by Weisiger, Hausmann & Craig (124) who isolated a sulphur-con-

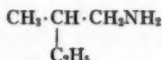
taining compound with the structure 2-(1-methylbutyryl) thiazole 4-carboxylic acid.



Furthermore, Lockhart and collaborators (140), on desulphurising bacitracin-A with Raney nickel have demonstrated the appearance of a new terminal alanyl group in the sulphur-free product and on hydrolysis of the latter have isolated isoleucinol



1-amino, 2-methyl-*n*-butane



and ethylamine.

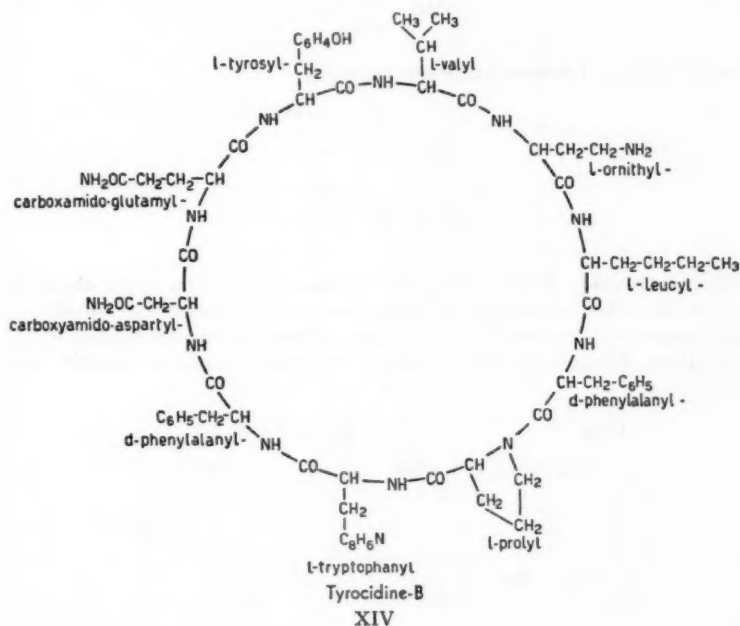
Some of the lysine-containing peptides obtained on hydrolysis of bacitracin-A are remarkably resistant to hydrolysis (141). The properties of one of these, Asp-Lys, suggested that it might not be a simple peptide. The dipeptide has now been synthesised (142) and the synthetic dipeptide is not identical with the hydrolysis product, but is converted to it by hot strong hydrochloric acid (143). Ring closure is postulated under these conditions. If this is the case, the dipeptide isolated by hydrolysis from bacitracin provides no evidence whether the  $\alpha$ - or the  $\beta$ -carboxyl group of the aspartic acid is involved in the peptide linkage in bacitracin-A itself.

*Tyrocidines*.—Tyrocidine-B, one of the three components of crude tyrocidine, has been purified by King & Craig (144, 145) and the complete sequence of amino acids in the molecule has been established (146) (XIV). Tyrocidine-B differs from tyrocidine-A in that it contains an L-tryptophan residue instead of a D-phenylalanine residue.

*Albomycin and grisein*.—A comparison (147, 148) of the chemical and biological properties of the iron containing polypeptide antibiotic albomycin (149) with those of the antibiotic grisein (150, 151) has shown them to be very similar, if not identical. Chromatographic studies show that albomycin consists of four antibacterial components, termed A, B, C and D which also are found in crude grisein. During the purification process compound C is converted into A.

*Actinomycins*.—Brockmann and his co-workers have made considerable further progress in the elucidation of the structure of the actinomycins. These are chromopeptides, containing a chromophore and a number of amino acids.

So far fifteen different actinomycins have been separated (152). The sequence of the amino acids in the peptide chain of actinomycins C<sub>2</sub> and C<sub>3</sub> (L-threonine, N-methyl L-valine, sarcosine L-proline, D-alloisoleucine) was estab-

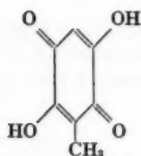


lished by hydrazinolysis (153) which led to the formation of N-methyl-valylsarcosine anhydride and L-prolyl-D-Valyl anhydride (actinomycin-C<sub>1</sub> contains 2 moles of D-valine instead of the two moles of D-allo-isoleucine present in actinomycin-C<sub>3</sub>) (154). The threonine moiety is linked directly to the chromophore actinocin. This was established through the isolation on vigorous acid hydrolysis of actinomycin-C (a mixture of actinomycins C<sub>1</sub>, C<sub>2</sub> and C<sub>3</sub>), of the red chromopeptide desamino-actinocyl-threonine, C<sub>22</sub>H<sub>22</sub>O<sub>8</sub>N<sub>3</sub>, which contains threonine as the sole amino acid (155) and which is very similar to actinocinin C<sub>15</sub>H<sub>11</sub>O<sub>8</sub>N, an amino acid-free acid degradation product of the actinomycins closely related to the chromophore.

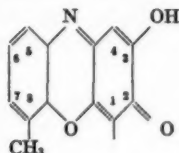
In addition to desamino-actinocyl threonine and actinocinin two other crystalline fragments were isolated from the products of vigorous acid hydrolysis of actinomycin-C which were recognized as 2,5 dihydroxytoluquinone



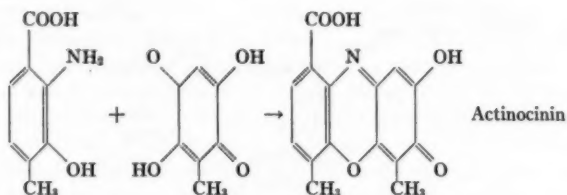
## CHAIN



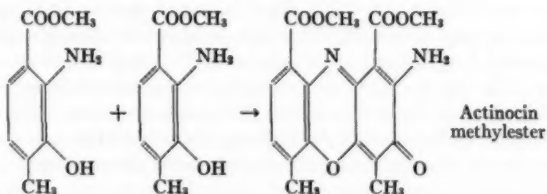
and 3-hydroxy, 1,8-dimethyl phenoxazone-2



(156) respectively. These findings led to the understanding of the chemical nature of actinocinin as a phenoxazone derivative (1,8-dimethyl, 3-hydroxy, phenoxazone-2,5 carboxylic acid); it was synthesised by condensation of 2,5 dihydroxy toluquinone and 2-amino, 3-hydroxy, 4-methyl benzoic acid (157)

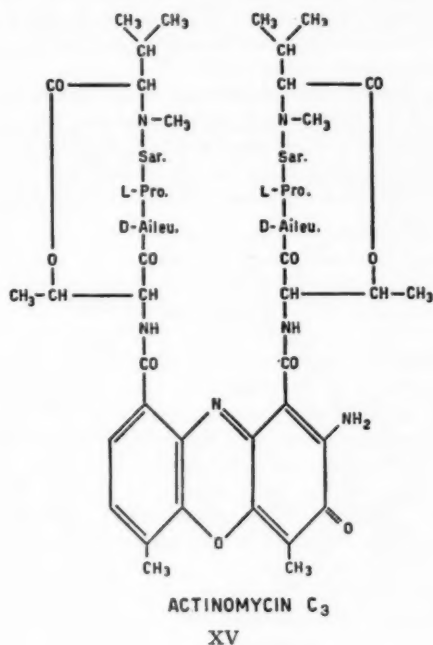


The chromophore of the actinomycins was recognized as 1,8-dimethyl, 3-amino, 4,5-dicarboxy phenoxazone-2 and its methylester synthesised by oxidative condensation of methyl 2-amino, 3-hydroxy, 4-methyl benzoate (158).

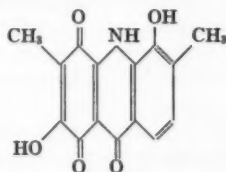


On treatment of actinomycin-C<sub>3</sub> with methanolic alkali two acid groups were liberated which were recognized to form part of a lactone ring, the hydroxyl

group belonging to the threonine, the carboxyl group to the N-methyl valine moieties (159). On the basis of these facts structural formula XV (160) was



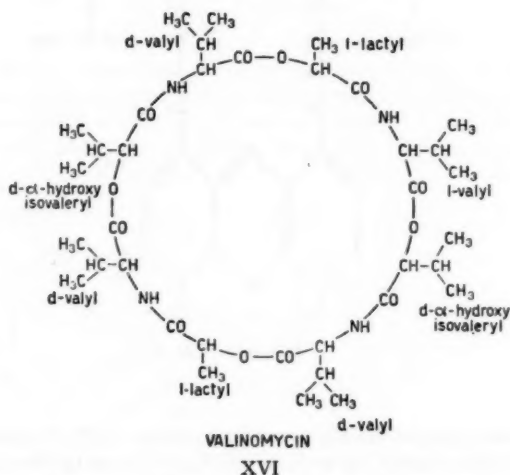
suggested for actinomycin-C<sub>3</sub>; in actinomycin-C<sub>1</sub> both alioisoleucine parts are replaced by D-valine, in actinomycin-C<sub>2</sub> one mole of alioisoleucine. On treatment of the actinomycins with baryta, an amino acid-free isomer of actinocinin, termed despeptido actinomycin, was obtained(161).



This was recognized as 3,6-dimethyl, 4,7-dihydroxy acridone-quinone-(5,8) and synthesised (162). The mechanism of this arrangement of the phenoxazine ring of the chromophore into an acridine ring, apparently involving

cleavage at the hetero oxygen atom and recondensation of the benzenoid and quinoid rings through the benzenoid carboxyl group, is as yet not understood. In this connection the isolation of 7-methyl benzoxazolone-4-carboxylic acid (structure confirmed by synthesis by alkaline peroxide oxidation of 6-methyl, 7-hydroxyisatin) from the products of alkaline peroxide degradation of actinomycin is of interest (166).

By adding D,L-isoleucine and sarcosine to the culture medium as actinomycin precursors the type of actinomycin produced may be influenced and new biosynthetic actinomycins may be obtained (163). A method of purifying mixtures of actinomycins by means of partition chromatography on cellulose columns has been described (164). For classification of actinomycin complexes see (165).



*Valinomycin*.—This antibiotic, produced by *Streptococcus fulvissimus* and active *in vitro* against the tubercle bacillus, was isolated in the crystalline state by Brockman & Schmidt-Kastner (167). It has the composition  $C_{60}H_{104}O_{12}N_4$  and yields on hydrolysis two moles of D-valine, two moles of L-valine, two moles of L-lactic acid and two moles of D-α-hydroxy-isovaleric acid (168).

Partial hydrolysis yielded L-lactyl-L-valine and D-α-hydroxyisovaleryl-D-valine, both obtained crystalline in form of their lactones. These findings and other considerations based on the results of partial hydrolysis of valinomycin led to its formulation as XVI.

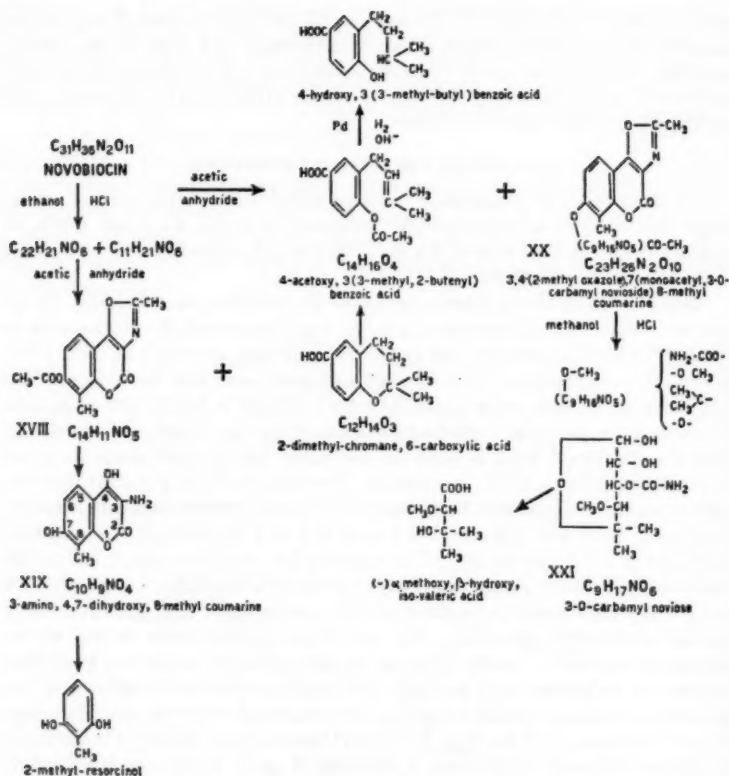
*Mode of action of polymixins*.—Newton has prepared a fluorescent polymixin by coupling this antibiotic with 1-dimethyl-amino naphthalene-5-sulphonyl chloride, the γ-group of the α,γ-diamino butyric acid in the poly-

mixin molecule reacting with this reagent. He followed the fate of polymixin-sensitive bacteria under the fluorescent microscope and came to the conclusion that the antibiotic acted by disorganizing the cell membrane (168a). For reviews of mode of action of the polymixins see (168b) and of polymixins and other polypeptide antibiotics (168c).

#### ANTIBIOTICS OF GLYCOSIDE NATURE

*Streptothricin and streptolin-B.*—These antibiotics (61) contain an amino sugar identified as  $\alpha$ -D-gulosamine (2-amino-2 deoxy- $\alpha$ -D-gulose) (169). It appears to be the first time that a sugar of the gulose configuration has been discovered in nature (169).

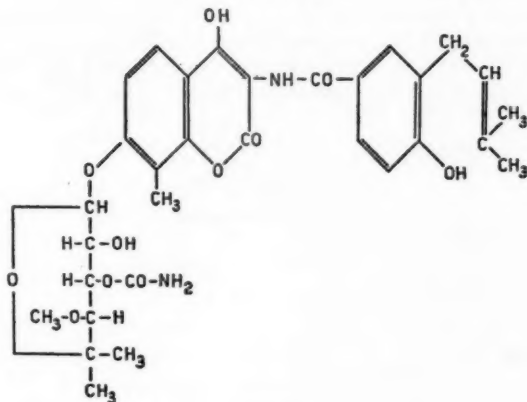
*Novobiocin.*—This antibiotic, produced by *Streptomyces spheroides*, *Streptomyces niveus*, and *Streptomyces griseus*, was discovered simultaneously in three different laboratories and given five different names (170, 171, 172): albamycin, cathomycin, cardelmycin, streptonivicin, and novobiocin. The last name seems now to be generally used (173). It is highly active against Gram-positive bacteria including penicillin-resistant *Staphylococci* and on antibacterial blood level is kept up for many hours after doses as small as 10 mg/k (174 to 177). It appears, however, to have the drawback to induce readily resistance in *Staphylococci* (178). Novobiocin is a colourless dibasic acid with pK values of 4.3 and 9.1 and elementary composition  $C_{22}H_{21}N_2O_{11}$  (172, 179 to 182). The degradation reactions which led to the elucidation of its structure are presented in fig. XVIII–XXI. Acetolysis with acetic anhydride gives two split products, a monobasic acid  $C_{14}H_{16}O_4$  and a neutral substance  $C_{22}H_{21}N_2O_{10}$ . The acid  $C_{14}H_{16}O_4$  was shown to contain an acetoxy group and a double bond in its side chain in 2-position; it yielded acetone on oxidation with periodic acid and osmium oxide indicating the presence of an iso-propylidene group. It was proved to be the acetyl derivative of 4-hydroxy, 3-(3-methyl, 2-butenyl) benzoic acid (XVII). On catalytic reduction followed by alkaline hydrolysis it gave 4-hydroxy, 3-(3-methylbutyl) benzoic acid. On ethanolysis (179) novobiocin gave two substances  $C_{22}H_{21}NO_6$  and  $C_{11}H_{21}NO_6$ . The latter was recognized as the ethyl glucoside of a sugar termed noviose (180) or novobiose (182). The former,  $C_{22}H_{21}NO_6$ , on acetylation with acetic anhydride, gave an acid  $C_{12}H_{14}O_3$  which was recognized as 2-dimethylchromane 6-carboxylic acid and could be converted with acetic anhydride to XVII, and a neutral product  $C_{14}H_{11}NO_5$ . The latter, on deacetylation gave the amphoteric substance  $C_{10}H_9NO_4$  which by reacetylation could be reconverted to  $C_{14}H_{11}NO_5$ . The behaviour of  $C_{10}H_9NO_4$  on acetylation, particularly with regard to the ultraviolet absorption spectra, suggested that it was a 3-amino, 4-hydroxy coumarine with a second hydroxyl and methyl substituent group in the benzene ring, and that  $C_{14}H_{11}NO_5$  was the acetoxy-3, 4-isoxazole derivative XVIII. The position of the substituent groups was located in 7 for the second hydroxyl and 8 for the methyl groups by degradation to 2-methyl resorcinol from which it followed that the formula for  $C_{10}H_9NO_4$  (XIX) and for  $C_{14}H_{11}NO_5$  were those given in fig.



## XVIII-XXI

XVIII-XXI. The moiety XIX is linked with XVII (see text) in the novobiocin molecule by the amino and carboxyl groups, respectively, the noviose moiety glycosidically to the 7-hydroxyl group of the coumarine moiety. The structure of the sugar moiety was elucidated (180) by degradation of the acetolysis product  $\text{C}_{23}\text{H}_{26}\text{N}_2\text{O}_{10}$ . This product proved to be the monoacetyl novioside XX which on methanolysis yielded a methyl monoacetyl novioside. On treatment with alkali (181) it split off  $\text{CO}_2$  and ammonia, suggesting the presence of a carbamyl grouping and accounting for the nitrogen and for two of the five oxygens of the noviose molecule. One of the remaining three oxygens was present in a hydroxyl group, the other in form of a methoxyl group and the third in form of the oxygen bridge. The presence of a gem-dimethyl group in the noviose molecule was established. Periodate oxidation localized the carbamyl group in 3-position and led,

## NOVOBIOCIN



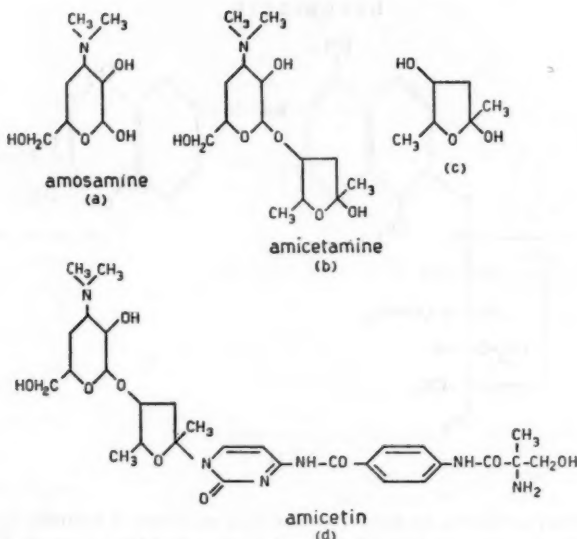
XXII

among other products, to the formation of  $\alpha$ -methoxy,  $\beta$ -hydroxy isovaleric acid (181); this showed that 3-barbamyl-noviose had the structure XXI and was an L-lyxose derivative. The complete structure of novobiocin is represented by formula XXII. The experimental details of the degradation reaction have been published by Hinman, Caron & Hoeksema (183).

**Amicetin.**—This antibiotic, produced by a streptomycete, has the empirical composition  $C_{29}H_{42}N_4O_9$  (61) and is a derivative of a new dimethylamino sugar, amosamine XXIIIa (184). On acid hydrolysis amicetin, in addition to  $\alpha$ -methylserine, *p*-aminobenzoic acid, and cytosine (185), gives a glycoside  $C_{14}H_{27}NO_6$ , amicetamine. Periodate oxidation of amicetamine gave glyoxal and formaldehyde, hydrolysis by acid resin, amosamine hydrochloride  $C_8H_{17}NO_4 \cdot HCl$ . On oxidation of amosamine with periodate one mole of formaldehyde and two of formic acid were formed suggesting, in conjunction with the formation of glyoxal from amicetamine, a 4-desoxy sugar structure for amosamine. The dimethylamino group was located in position 3 and, on the basis of these findings structure XXIIIa was deduced for amosamine. The neutral moiety of amicetamine was obtained by methanolysis of amicetin. The resulting methyl glycoside did not react with periodic acid, but on oxidation of the free ketose with this reagent over 50 per cent of acetaldehyde was formed. Structure XXIIIc was therefore deduced for the ketose moiety. As the cytosine moiety was shown to be attached to the ketose moiety through the ketal carbon by the iodoform test, the amino sugar must be attached through its aldehyde group to the only other hydroxyl group of the ketose moiety to form the nonreducing glycoside amicetamine XXIIIb. The complete structure for amicetin was therefore deduced as XXIIId.

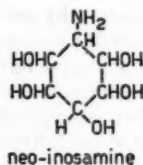
**Hygromycin.**—Degradation by acid and alkali (186) of this antibiotic





XXIII

produced by *Streptomyces hygroscopicus* and active against Gram-positive and Gram-negative bacteria, has shown that it is built up from three main units: 3,4-dihydroxy  $\alpha$ -methyl cinnamic acid, an inosamine and 5-keto-6-deoxy-D-arabohexose. The inosamine, obtained as the crystalline hydrochloride, was tentatively identified as the hitherto unknown neoinosamine-2 (XXIV). The inosamine and 3,4-dihydroxy,  $\alpha$ -methyl cinnamic acid moieties

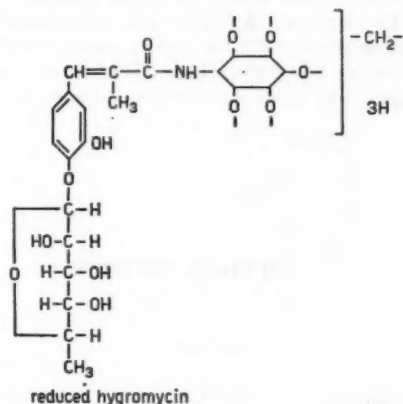


XXIV

were shown to be linked by the amino and carboxyl groups respectively. The presence of an additional carbon atom was revealed by analysis of a crystalline derivative of the amide and by the formation of formaldehyde from it. This carbon atom must therefore be present as a dioxy methylene group linking two of the hydroxyl groups of the inosamine moiety. The exact position of this methylene linkage has not yet been established.

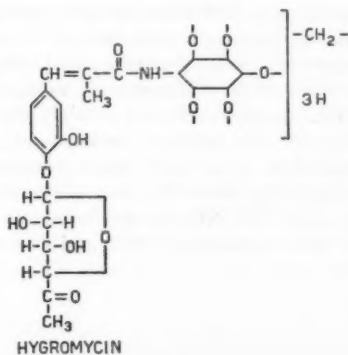
On methylation of hygromycin and subsequent alkaline analysis 4-hydroxy, 3-methoxy,  $\alpha$ -methyl cinnamic acid was obtained. This showed that the linkage between the sugar and cinnamic acid moieties occurred

through the hydroxyl group in 4-position. The sugar moiety was obtained by mercaptonolysis of sodium borohydride-reduced hygromycin as diethyl mercapto-L-fucose; the structure of reduced hygromycin is therefore XXV. The free carbonyl group in hygromycin was shown to be in 5-position of the sugar moiety by periodate degradation of a mercaptal obtained by



XXV

mercaptan treatment of hygromycin (which gave a product no longer giving carbonyl reactions, presumably the mercaptol) followed by desulphurisation with Raney nickel (in which the carbonyl group was reduced to a methylene group) and mercaptolysis (leading to the diethyl mercaptal of the deoxy sugar). This mercaptal gave propionaldehyde in 70 per cent yield establishing the structure of the sugar moiety in hygromycin as 5-keto-6-deoxy arabo hexose. On the basis of these findings structure XXVI was assigned to hygromycin.

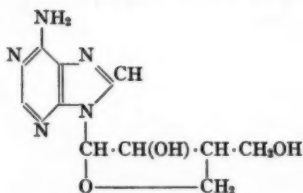


XXVI

Several antibiotics of nucleoside nature are now known.

**Nebularin.**—Nebularin, an antibiotic active against mycobacteria and isolated in the crystalline state from *Agaricus (Clitocybe) nebularis*, of the composition  $C_{10}H_{12}N_4O_4$ , was shown to be 9-( $\beta$ -D-ribofuranosyl) purine (187). This was synthesised by Brown & Weliky (188).

**Cordycepin.**—A crystalline antibiotic active against *B. subtilis*, termed cordycepin, with the composition  $C_{10}H_{13}N_5O_3$  was isolated from culture filtrates of *Cordyceps militaris* (189). On acid degradation it gave adenine and a sugar, termed cordycepose (190), which has a structure related to apiose. The structure assigned to cordycepin is 9-cordyceposyl-adenine (XXVII).



XXVII

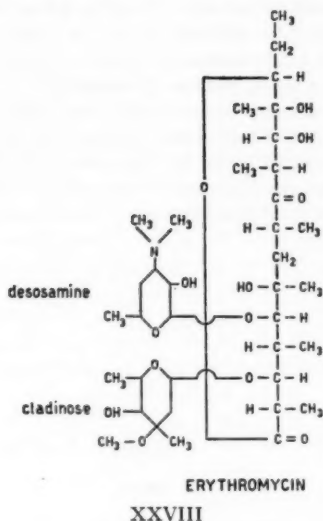
**Nucleocidin.**—Nucleocidin,  $C_{11}H_{16}N_4SO_4$  is an antibiotic produced by a *Streptomyces* strain found in Dinepur (India) soil and, like puromycin (61), is active against trypanosomes, in addition to a number of Gram-positive and Gram-negative bacteria (42). In a recent preliminary note nucleocidin was shown to be a glucoside of adenine in which the carbohydrate moiety, stated to be of unusual structure, is bound in ester linkage to sulphamic acid (191).

#### ANTIBIOTICS CONTAINING A MULTIMEMBERED LACTONE RING (MACROLIDES)

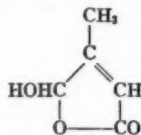
Some of the more recent antibiotics have been shown to possess the common structural feature of being derivatives of a dimethylamino sugar, desosamine, and an aglucone of multimembered lactone structure. Termed macrolides (203), they represent a novel class of natural compounds, of unusual biochemical interest, and include erythromycin, methymycin, narbomycin, picromycin, carbomycin, oleandomycin and possibly the foromacidsins.

**Erythromycin.**—In a series of brilliant investigations Flynn and his collaborators have elucidated the complete chemical structure of erythromycin.

Erythromycin,  $C_{27}H_{47}NO_{13}$  (XXVIII), is a bisglycoside composed of a dimethylamino-deoxy sugar  $C_8H_{17}NO_3$ , termed desosamine (192), a methoxy-deoxy sugar  $C_8H_{16}O_4$  termed cladinose (192) and the aglucone  $C_{11}H_{19}O_6$ , termed



erythronolide (193, 194). The structure of desosamine had previously been established (192). Cladinose, which was shown to contain one methoxy, two hydroxy and two C-methyl groups (195) and on oxidative degradation gave, among other products,  $\beta$ -formyl crotonic acid



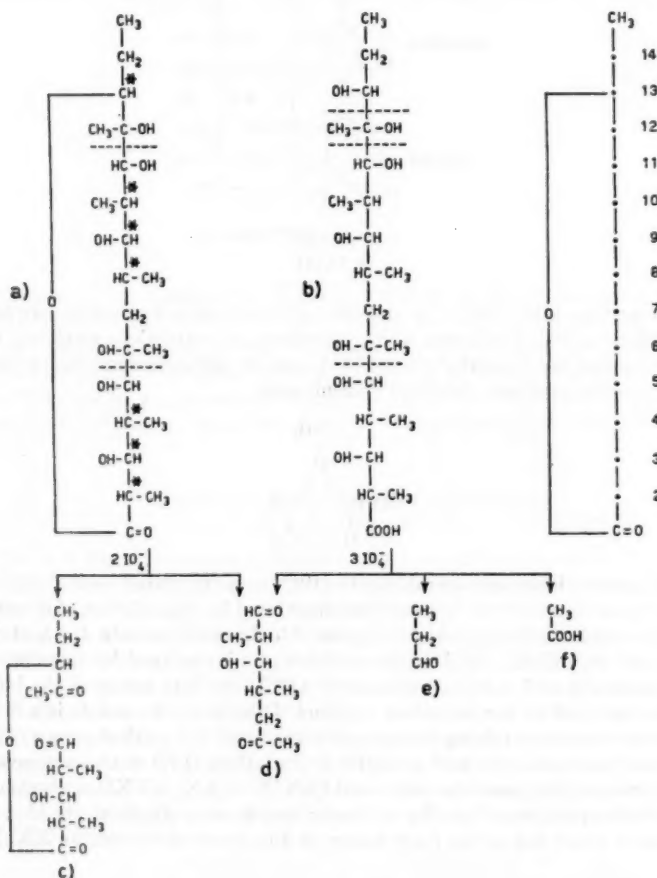
in its pseudo form and acetaldehyde (196), was elucidated as in XXVIII.

The structure of the aglucone was elucidated by degradation of dihydroerythronolide  $C_{21}H_{40}O_8$ , obtained from dihydroerythromycin by acid hydrolysis (193) (XXIXa). Dihydroerythromycin is obtained by reduction of erythromycin with sodium borohydride (193); the keto group of the latter is thus reduced to the secondary carbinol. Dihydroerythronolide is a hexahydroxylactone containing two glycol systems and 7 C-methyl groups (194). By a series of reductive and oxidative degradations (197) of the lactone and the corresponding pentadecanoic acid (XXIX, XXX, XXXI) a number of breakdown products of smaller molecular weight were obtained, the identification of which led to the formulation of dihydroerythronolide as XXIXa.

Periodate degradation of dihydroerythronolide led to the  $C_{12}$ -keto-aldehyde-hydroxy ester XXIXc (also XXXc) which, on alkaline hydrolysis yielded pentane-3-ol, 2-one, XXXg, representing its keto moiety.

The reduced keto moiety, pentane-2,3-diol XXXh, was obtained among the products of catalytic reduction of XXXc; on oxidation with bromine, followed by treatment with 2,4-dinitrophenylhydrazine, it gave the same 2,4-dinitrophenylhydrazone XXXi as the keto moiety itself.

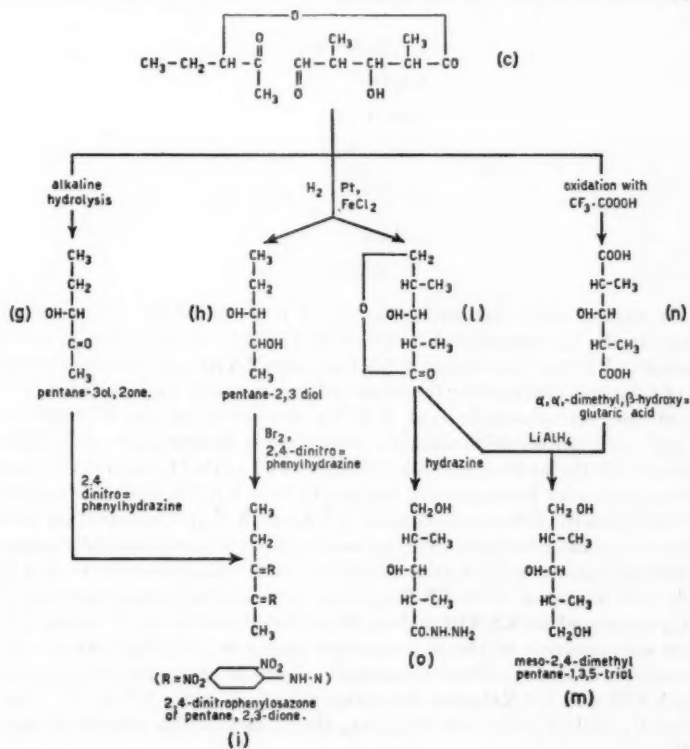
A derivative of the aldehyde-acid moiety of XXXc was obtained, also among the products of catalytic reduction of XXXc, as the hydroxy lactone



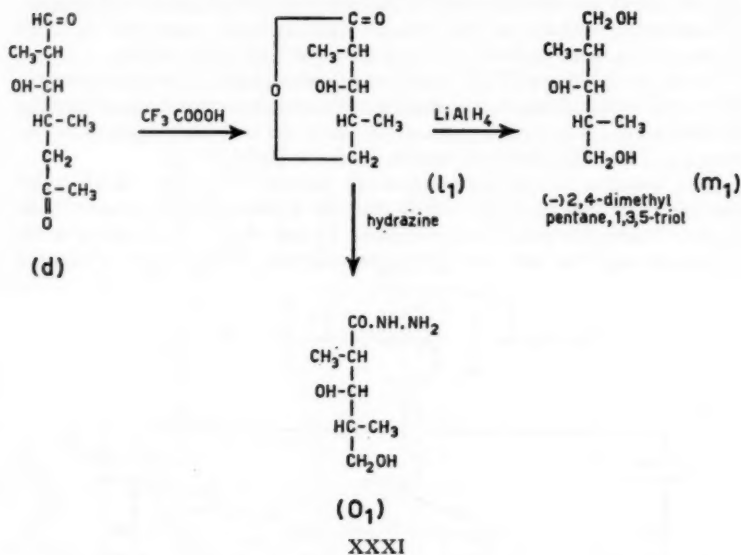
XXXI; this lactone could be transformed by reduction with lithium aluminium hydride into meso-2,4 dimethyl pentane-1,3,5-triol XXXm, identified by comparison with an authentic specimen prepared by lithium aluminium hydride reduction of synthetic  $\alpha\alpha$ -dimethyl,  $\beta$ -hydroxyglutaric acid. This acid (XXXn) was isolated among the products of oxidation with peroxytrifluoroacetic acid of the  $C_{12}$ -keto-aldehyde-hydroxy ester XXXc. With hydrazine the hydroxylactone XXXI gave the hydrazide XXXo.

As hydroxylactone XXXI contains a carboxyl group it represents the first seven atoms of dihydroerythronolide; as it can be transformed into the meso-forms of 2,4-dimethyl pentane-1,3,5-triol the two methyl groups at carbons 2 and 4 of dihydroerythronolide are in *cis*-position.

The isolation of the hydroxyketone pentane-3-ol,2-one (XXXg) by alkaline hydrolysis of XXXc shows that the lactone linkage present in dihydroerythronolide must involve carbon 13 and that it is therefore a 14-membered ring. The fact that the hydroxyketone XXXg could be isolated



from a periodate oxidation product of dihydroerythronolide by simple alkaline hydrolysis (it had survived periodate oxidation), proves that the carbons adjacent to carbons 13, carrying the oxygen of the lactone linkage, i.e., carbons 11 and 12, must be part of a glycol system.

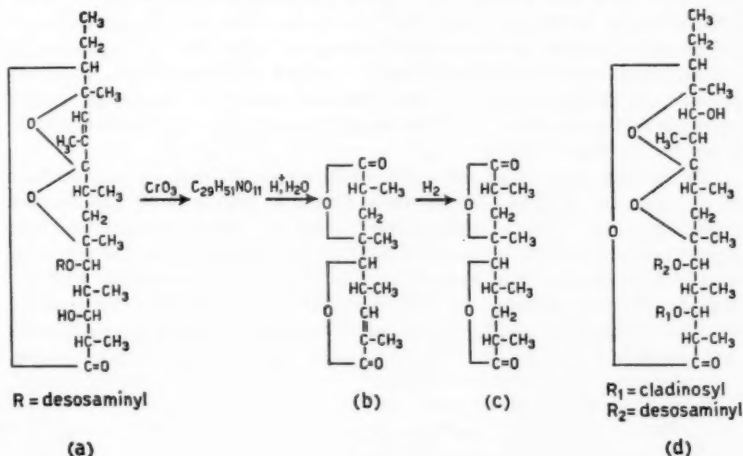


The carbon chain comprising carbons 6 to 11 of dihydroerythronolide appears in the  $\text{C}_9$  compound XXIXd (also XXXId) which is formed simultaneously with the  $\text{C}_{12}$ -compound XXIXc (also XXXc) on periodate oxidation of the pentadecanoic acid XXIXb. Oxidation of the  $\text{C}_9$ -fragment XXXId with peroxy-trifluoroacetic acid gives a hydroxylactone XXXII<sub>1</sub> isomeric with the hydroxylactone XXXI, as well as its O-acetyl derivative. On treatment with hydrazine the hydroxylactone XXXII<sub>1</sub> gave a hydrazide XXXIo<sub>1</sub> isomeric with the hydrazide XXXo of XXXI. On reduction with lithium aluminium hydride, hydroxylactone XXXII<sub>1</sub> gives optically active 2,4-dimethyl pentane-1,3,5 triol XXXIm<sub>1</sub> in contradistinction to XXXI which gives the meso form XXXm. These facts establish the structures of the  $\text{C}_9$ -compound as XXXId and of dihydroerythronolide as XXIXa. The optical configuration of the seven carbon atoms of XXIXa marked with an asterisk was deduced from the optical behaviour of the two hydroxylactones XXXI and XXXII<sub>1</sub> and their respective hydrazides XXXo, XXXIo<sub>1</sub>, the two  $\text{C}_7$  triols XXXm and XXXIm<sub>1</sub>, the  $\text{C}_8$  diol XXXh and the  $\text{C}_7$ -acid XXXn.

The dihydroerythronolide molecule contains the striking feature that a



three carbon unit repeats itself seven times in regular recurrence. This suggests that the molecule may be built up biologically from propionic acid residues by "head-to-centre" linkages; other branched long chain compounds found in biological materials, as for instance the tubercle acids, tuberculo-stearic and mycoceranic acids may have a similar pattern of biosynthesis. The position of the sugar moieties of the carbonyl group and the size of the lactone ring was elucidated by Wiley *et al.* (196). Isolation of the hydroxy pentanone XXX by periodate oxidation of erythromycin-N-oxide (192) showed that the lactone ring in erythromycin has the same size as in dihydro-erythronolide, i.e. terminates at C-13. Oxidation with chromium oxide of erythralosamine, an acid hydrolysis product of erythromycin in which the cladinose portion was removed (192, 194), followed by acid hydrolysis led to the unsaturated dilactone  $C_{13}H_{20}O_4$  (198). Infrared analysis and oxidative degradation established structure XXXIIc for the saturated and XXXIIb for the unsaturated dilactone. The dilactone contains one of the vicinal



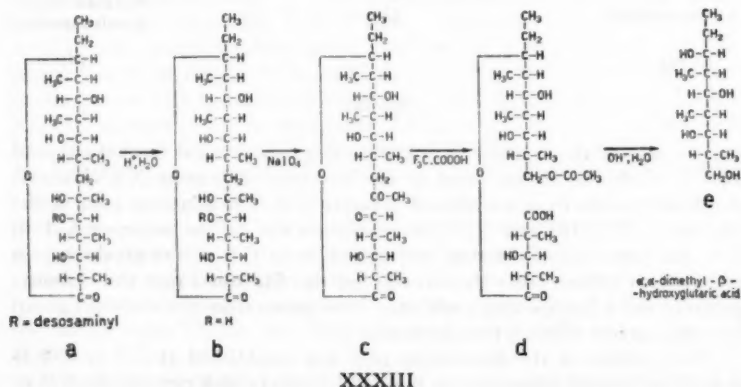
## XXXII

oxygen pairs of the erythromycin lactone ring, which must be that situated at C-5—C-6; the double bond in the unsaturated lactone XXXIIb evidently originates from a hydroxyl group in C-3. The  $\delta$ -lactone part of the dilactones XXXIIb and XXXIIc represents the carbon sequence C-1 to C-5, the  $\gamma$ -lactone the carbon sequence C-6 to C-9 of the erythromycin molecule. It follows from the structure of the dilactones that the carbonyl group of the  $\gamma$ -lactone ring could only have arisen from the carbonyl group in erythromycin which is thus located at C-9.

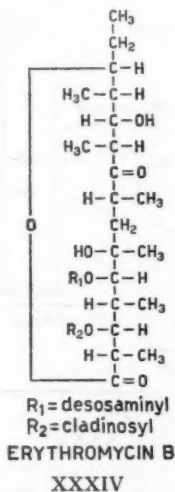
The position of the desosamine part was established at C-5 or C-6 as it must be located adjacent to a hydroxyl group (which can only be C-5 or

C-6, the only other possibility, C-11 or C-12, being eliminated). Desosamine is eliminated by vigorous alkaline hydrolysis of erythrolosamine which can be explained only by the fact that it must be situated in a position in which an adjacent hydrogen could be activated under these conditions. Only position 5 fulfills these requirements. The structure of erythrolosamine is given by XXXIIa. It is formed from erythromycin with the loss of cladinose, two moles of water and the ketonic group and contains only two hydroxyl groups one of which is present in the desosamine moiety. The isolation of dilactone XXXIIb from erythrolosamine shows that the carbon chain C-1 to C-9 in erythromycin has undergone no rearrangement. If the hydroxyl group present in erythrolosamine at C-3 is eliminated by alkali and  $\alpha, \beta$  unsaturated lactone would be formed which would activate a hydrogen atom at C-4 leading to the elimination of desosamine from C-5. The presence of a hydroxyl group at C-6 is also indicated by the facile spiroketal formation when erythromycin is kept at pH 2.5 for a few minutes or pH 4 for a few hours. Under these conditions anhydroerythromycin (XXXIIId) is formed in which the ketonic function has disappeared and spiroketal formation, with the loss of one mole of water, has taken place with the participation of the hydroxyl groups located at C-6 and C-12. The only possible remaining position for the cladinose moiety in the erythromycin molecule is therefore at C-3. The complete structure for erythromycin is given by formula XXVIII.

*Erythromycin-B.*—A second antibiotic was isolated from the culture fluid of *Streptococcus erythreus* by Pettinga, Shark & Abele (199) and termed erythromycin-B, with the composition  $C_{27}H_{47}NO_{13}$ . In its chemical and biological properties (200) it closely resembled erythromycin but did not form readily a spiroketal. Mild hydrolysis yielded cladinose. Reduction with sodium borohydride, followed by mild acid hydrolysis yielded 5-O-desosaminyl dihydroerythronolide-B (XXXIIIa) which on acid hydrolysis gave dihydroerythronolide-B (XXXIIIb). This compound consumed one mole of sodium

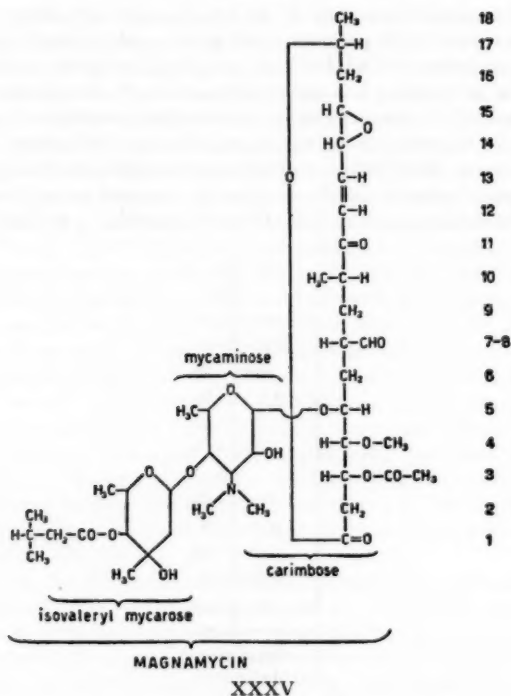


periodate, in contradistinction to 5-O-desosaminyl dihydroerythronolide which did not react with periodate, and gave an aldehyde keto ester, presumably of structure XXXIIIc. This compound on further oxidation with trifluoroacetic acid, gave a keto ester acid, presumably of structure XXXIIId which on hydrolysis gave meso,  $\alpha$ ,  $\alpha^1$ -dimethyl- $\beta$ -hydroxy glutaric acid (XXXn) and a  $C_{12}$ -tetrol of the structure XXXIIIe. This allows the assignment of structure XXXIIIb for dihydroerythronolide-B. The positions of the two sugars cladinose and desosamine are assumed to be the same as in erythromycin, hence structure XXXIV for erythromycin-B (201).



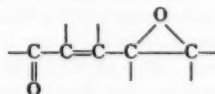
*Erythromycin-C*.—A third crystalline antibiotic similar in properties to erythromycin was isolated from the fermentation broth of certain strains of *Streptococcus erythreus* and termed erythromycin-C. Like erythromycin, it yielded on hydrolysis erythrolosamine and 5-O-desosaminyl dihydroerythronolide, but instead of cladinose another neutral sugar was obtained. The structure of erythromycin-C is assumed to be the same as that of erythromycin except for the neutral sugar moiety (202).

*Magnamycin (carbomycin)*.—A review of the structural studies on magnamycin,  $C_{42}H_{67}O_{16}N$ , has recently been published by Woodward (203). It consists of an 18-membered lactone linked to a disaccharide. The latter is built up from the dimethylamino sugar mycaminose (204) (XXXV) and the desoxy sugar mycarose (205) (XXXV) in which one of the hydroxyl groups is acetylated by isovaleric acid. The mycarose moiety is obtained in form of methyl isovaleryl mycaroside by mild methanolysis of magnamycin (204). Under these conditions the mycaminose moiety is obtained attached to the lactone moiety of magnamycin as a basic glycoside termed carimbose

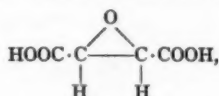


from which it can be isolated in the free form after vigorous hydrolysis. Which of the hydroxyl groups of mycaminoside is involved in the linkage to the lactone moiety was established by exhaustive methylation of magnamycin with silver oxide and methyl iodide and subsequent hydrolysis; this gave an O-methyl-mycaminoside methochloride which on oxidation with periodate yielded acetaldehyde and no formic acid.

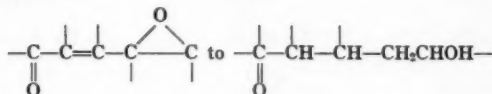
The nature of the eight oxygen atoms of the lactone moiety  $C_{22}H_{32}O_8$  was as follows: one in a methoxyl, two in an acetyl, one in a free aldehyde group (giving an oxime, a thiosemicarbazone, a dimethylacetal and, on oxidation, a carboxyl function). Two further oxygen atoms were present in the grouping:



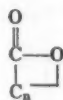
i.e., in form of an ethylene oxide linked to an  $\alpha, \beta$ -unsaturated keto function. This became evident from the absorption spectrum, isolation of ethylene oxide dicarboxylic acid,



on oxidation of magnamycin with dilute nitric acid, and the formation of a diacetate on acetylation of carimbose, but of a triacetate on acetylation of tetrahydrocarimbose (formed by catalytic hydrogenation of carimbose), indicating the change

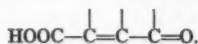


The remaining two oxygen atoms form a part of a lactone system

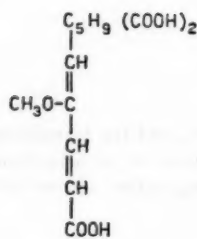


When magnamycin is treated with potassium iodide in acetic acid, iodine is liberated and the product  $\text{C}_{42}\text{H}_{67}\text{O}_{15}\text{N}$  is formed which differs from magnamycin by one oxygen atom and is characterized by the appearance of a new strong ultraviolet absorption band at  $278 \text{ m}\mu$ , which must be attributed to the  $\alpha$ -,  $\beta$ -,  $\gamma$ -, doubly unsaturated carbonyl system  $\text{CO} \text{---} \text{C}=\text{C} \text{---} \text{C}=\text{C}$ -, originating from the elimination of the ethylene oxide oxygen. The product  $\text{C}_{42}\text{H}_{67}\text{O}_{15}\text{N}$  accompanies natural magnamycin and has been termed magnamycin-b (206).

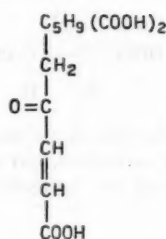
Oxidative degradation of magnamycin or carimbose with periodate, followed by treatment with hot aqueous potash, led to the isolation of the methoxy acid  $\text{C}_{13}\text{H}_{18}\text{O}_7$ , which, on hydrolysis with mineral acids, readily lost the methoxy group with the formation of the acid  $\text{C}_{12}\text{H}_{16}\text{O}_7$ . This acid was shown to contain the  $\alpha$ -,  $\beta$ -unsaturated  $\gamma$ -keto acid system,



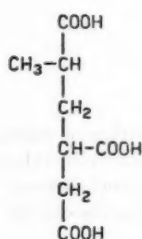
Both the  $\text{C}_{13}$  and the  $\text{C}_{12}$  acids were converted into a tribasic acid  $\text{C}_8\text{H}_{13}\text{O}_6$ , the former by ozonization followed by hydrogen peroxide oxidation, the latter by oxidation with dilute nitric acid; they therefore must have the partial structures XXXVIa and XXXVIb respectively. The acid  $\text{C}_8\text{H}_{13}\text{O}_6$  was shown to possess structure XXXVIc by synthesis through condensation of methyl malonate and itaconate, followed by hydrolysis and decarboxylation. By boiling with aqueous potash the  $\alpha$ -,  $\beta$ -unsaturated  $\gamma$ -keto acid XXXVIb was, in characteristic manner for the  $\alpha$ -,  $\beta$ -unsaturated carboxyl system, converted into the dibasic saturated keto acid XXXVIId, which on Wolff-Kishner reduction gave a dibasic acid XXXVIe recognized as an  $\alpha$ ,  $\alpha'$ -substituted glutaric acid with the structure XXXVIIf. It follows that

 $\text{C}_{13}\text{H}_{18}\text{O}_7$ 

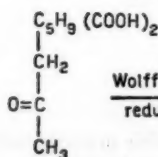
(a)

 $\text{C}_{12}\text{H}_{16}\text{O}_7$ 

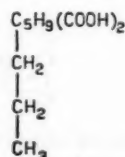
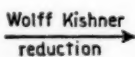
(b)

 $\text{C}_8\text{H}_{12}\text{O}_6$ 

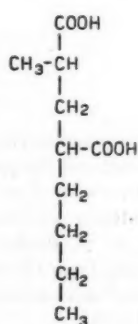
(c)



(d)



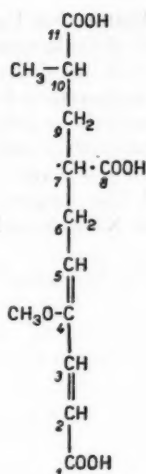
(e)



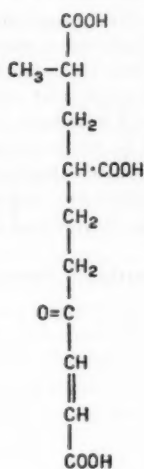
(f)

## XXXVI

the  $\text{C}_{13}$  methoxy acid and the  $\text{C}_{12}\alpha,\beta$ -unsaturated  $\gamma$ -keto acids must have structures XXXVIIa and XXXVIIb, respectively. Hence the partial structure XXXVIIc can be derived for magnamycin. This contains a free aldehyde and a keto group. The aldehyde group was located as attached to carbon 7 (XXXVIIc) because of the ease with which the carboxyl function derived from it by oxidation (after reduction of carimbose dimethyl acetal with lithium aluminium hydride, hydrogenation, hydrolysis to remove the acetal group and treatment with hydrogen peroxide) formed a five-membered lactone ring with the hydroxyl group appearing after removal of mycaminose by vigorous hydrolysis, while no lactone formation was observed with the mycaminose moiety attached. Lactone formation was also observed after exhaustive methylation of carimbose dimethyl acetal with methyl iodide and silver oxide, followed by hydrolysis and oxidation. These reactions, simul-

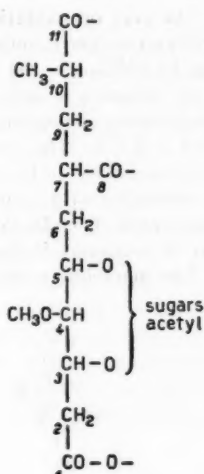


(a)



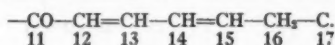
(b)

XXXVII

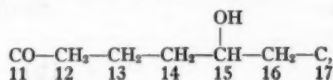


(c)

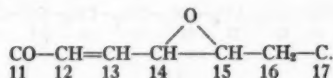
taneously with locating the aldehyde group at C-8, also locate the position of the disaccharide side chain at C-5 and the acetyl group at C-3. On the basis of these findings the structures for magnamycin and magnamycin-b can be expanded to XXXVIIIa and b. On oxidation of tetrahydromagnamycin-b with boiling nitric acid pimelic acid was isolated, showing that the molecule contained a sequence of 5  $\text{CH}_2$  groups. The sequence of carbon atoms of magnamycin-b, starting from  $\text{C}_{11}$ , is therefore



Correspondingly oxidation of tetrahydromagnamycin with boiling nitric acid gave glutaric acid, so that the sequence of carbon atoms in the tetrahydromagnamycin molecule, starting from  $\text{C}_{11}$ , is



and in the magnamycin molecule

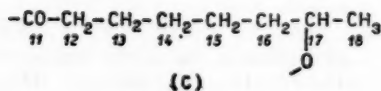
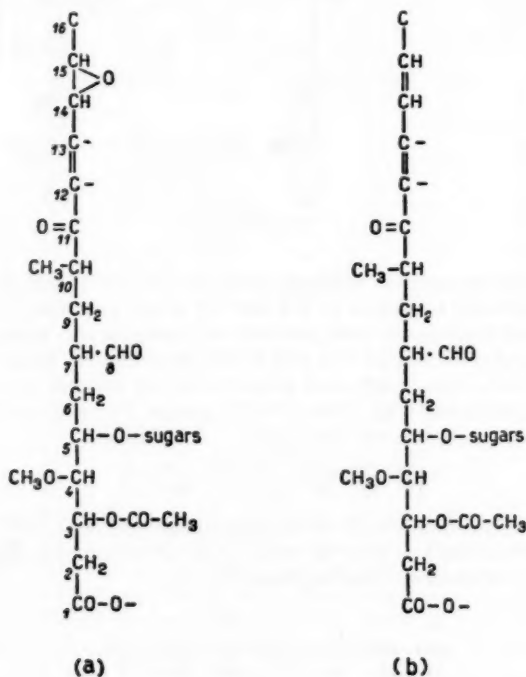


The keto group in magnamycin is thus located at C-11. The isolation of



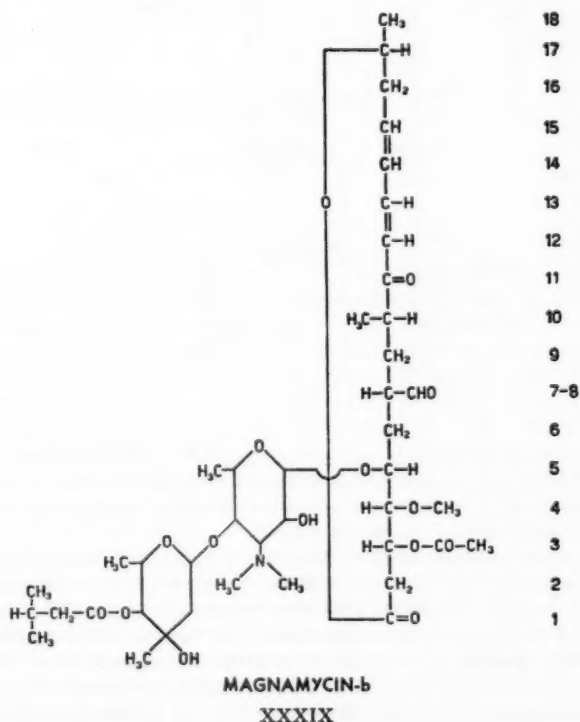
pimelic acid on oxidation of tetrahydromagnamycin-b indicates that C<sub>17</sub> carries an oxygen function which, of necessity, must form part of the lactone ring. In addition to the oxygen C-17 carries a methyl group; this was shown by the isolation of crotonic acid by nitric acid oxidation of magnamycin-b. The presence of sequence C-11—C-18 in tetrahydromagnamycin-b, as indicated in XXXVIIIc, was confirmed by the isolation of normal caprylic acid after ozonization of the enol acetate of tetrahydromagnamycin-b and vigorous reduction with hydrogen iodide and zinc and acetic acid. The complete magnamycin-b molecule is therefore represented by formula XXXIX and that of magnamycin by XXXV.

The macrolide molecule is of particular interest to the biochemist and



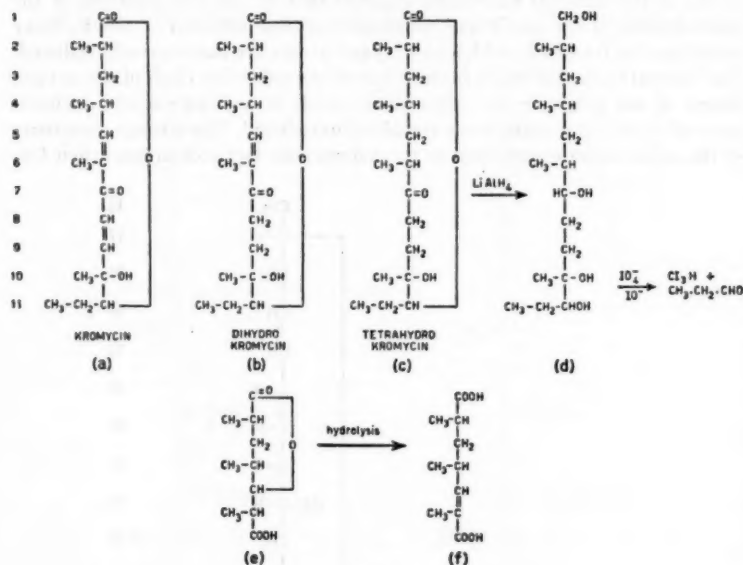
XXXVIII

deeper knowledge of these compounds may well lead to a more profound understanding of the general biosynthetic mechanisms in the fatty acid and sugar series. According to Woodward (203) the macrolides may be regarded in one respect as giant sugars, in others as long chain fatty acids. The macrocyclic lactones of some macrolides are obviously formed by condensation of active acetyl residues, in the same way that the normal fatty acids are formed, but in others, for instance erythromycin, propionyl residues are the condensing units, and in others still both acetyl and propionyl residues appear to take part in the condensation process. The distribution of the oxygen atoms in the different macrolides suggests that as the first products of the condensation of the acetic and propionic residues polyketo or polyhydroxy acids may be formed in which the oxygen atoms are subsequently reduced. The normal fatty acid chain is the result of the reduction of all of the oxygen atoms of the polyketo- or polyhydroxy acid; in the macrocyclic lactones some of the oxygen atoms have remained unreduced. The strange structures of the sugar moieties attached to the macrocyclic lactones suggest their for-



mation by condensation processes similar to those involved in the synthesis of fatty acids. Furthermore, Woodward points out that the macrolide sugars cladinose and mycarose closely resemble the unusual sugars of the steroidal glycosides; one of these, oleandrin, contains the same sugar, L-oleandrose, as the macrolide oleandomycin. Steroids, like the fatty acids and some of the macrolides are also known to be built up from acetyl residues, and Woodward suggests that the macrolides perhaps represent rudimentary steroid substitutes, formed by unicellular organisms.

*Picromycin.*—Previous studies by Brockmann and his collaborators (207



XL

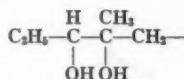
to 212) have shown that picromycin,  $C_{35}H_{48}O_7N$ , yielded on mild acid or alkaline hydrolysis the dimethylamino sugar picrocin, identical with desosamine, and an anhydro aglucone, cromycin. The structural studies were continued by a Swiss group of workers (213).

Elementary analyses of dihydrocromycin and tetrahydrocromycin, the crystalline hydrogenation products of cromycin, showed that this substance had the composition  $C_{17}H_{26}O_4$ . Dihydrocromycin was obtained by mild hydrolysis of dihydropicromycin (prepared by catalytic hydrogenation of picromycin), tetrahydrocromycin by catalytic hydrogenation of dihydrocromycin or cromycin. Ultraviolet and infrared spectroscopy of cromycin and dihydrocromycin indicate the presence of an  $\alpha,\beta$ -unsaturated ketone

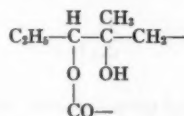
grouping, infrared spectroscopy, in addition, confirmed the presence of a lactone ring and a (tertiary) hydroxyl group previously established by Brockmann and co-workers.

On oxidation with potassium permanganate of picromycin the trimethyl-3 hydroxy-pimelic acid lactone XLe was obtained (214) which on hydrolysis and elimination of water gave the corresponding unsaturated trimethyl pimelic acid XLf. It obviously originated from carbons 1 to 7 [for numbering see (213)].

Treatment of dihydrocromycin with ozone and subsequent oxidative degradation of the ozonide with periodate and alkali led to the formation of meso  $\alpha,\alpha'$ -dimethylglutaric acid (214, originating from carbons 1 to 5), propionic aldehyde (originating from C-11 carrying the ethyl group) and levulinic acid (originating from carbon 7 to 10 carrying a methyl group). Reduction of tetrahydrocromycin with lithium aluminium hydride gave a tetrahydroxy-reduction product XLd which on oxidative degradation with periodate and hypiodite gave propionaldehyde and iodoform indicating the sequence

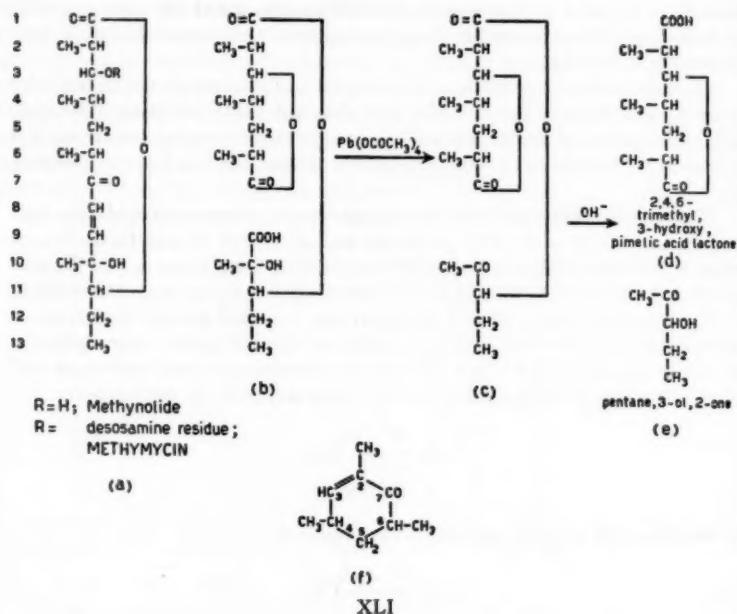


in the reduction product and hence the sequence



in cromycin and di- and tetrahydrocromycin. On the basis of these facts the formula XLc has been ascribed to tetrahydrocromycin and the formulae XLb and XLa to dihydrocromycin and cromycin, respectively. The desosamine moiety in picromycin is considered to be attached at carbon 5 (215).

**Methymycin.**—The structure of this antibiotic,  $\text{C}_{26}\text{H}_{43}\text{NO}_7$ , isolated from the culture fluid of a streptomycete (216) has been elucidated as the macrolide structure XLIa by Djerassi and co-workers (217, 218, 219). On hydrolysis with 5N HCl methymycin yielded the dimethylaminosugar desosamine (218). On mild hydrolysis with 5N  $\text{H}_2\text{SO}_4$  methynolide,  $\text{C}_{17}\text{H}_{29}\text{O}_6$ , was obtained. This contained a lactone, one keto and two hydroxyl groups. Methymycin itself was shown (220) to contain a lactone, an  $\alpha,\beta$ -unsaturated keto and two hydroxyl groups, one belonging to the desosamine moiety, the other to the methynolide moiety. On acetylation with pyridine-acetic anhydride methynolide gave readily a monoacetate; on oxidation with chromium trioxide, under conditions under which the hydroxyl group of methynolide in the intact methymycin molecule was resistant to this oxidizing agent, one of the two hydroxyls was converted into a keto group. Evidently the desosamine residue was linked in the methymycin molecule to methynolide

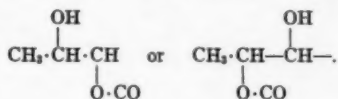


through this reactive hydroxyl group, shown to be in  $\beta$ -position to the lactone carboxyl, since the diketolactone obtained by chromium trioxide oxidation of methynolide liberated  $\text{CO}_2$  after saponification and oxidation. From the oxidation products of methynolide with permanganate, compounds XLIIb, XLIIc and XLIIId were isolated. XLIIc could also be obtained from XLIIb by oxidation with lead tetra-acetate. XLIIc, on alkaline hydrolysis, gave pentane-3-ol, 2-one XLIIe and the lactone acid XLIIId, which had also been isolated from the oxidative degradation products of picromycin and narbomycin (214), and was shown to be 2,4,6-trimethyl, 3-hydroxy pimelic acid lactone; the trimethylcyclo hexanone XLIIIf, obtained by alkali fusion of methymycin (218), evidently derives from the same carbon atoms 1 to 7 of the carbon chain of methynolide with the loss of the lactone carboxyl, as does XLIIId. On the basis of these observations structures XLIIb and XLIIc were assigned to the oxidative degradation products of methynolide and XLIIa to methynolide itself.

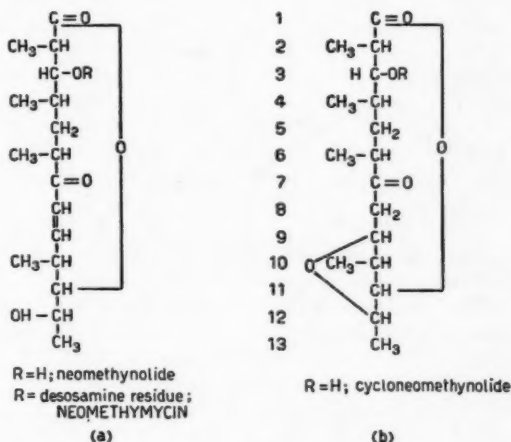
**Neomethymycin.**—A second antibiotic isomeric with methymycin and termed neomethymycin, was isolated from the methymycin fermentation mother liquors (40).

In contradistinction to methynolide, neomethynolide formed a diacetate, yielded acetaldehyde instead of propionaldehyde when treated successively

with lithium aluminium hydride and periodic acid, and gave a positive iodoform reaction. These facts indicate the presence of grouping



On ozonolysis neomethynolide yields the same lactone acid XLId as methymycin, thus establishing the same C-1 to C-7 sequence in neomethymycin as in methymycin. On cleavage with sulphuric acid cycloneomethynolide is obtained. This forms a monoacetate, does not give a positive iodoform test and does not react with periodic acid after lithium aluminium hydride reduction, indicating in cycloneomethynolide a new ether linkage formed by the addition of the hydroxyl group at C-12 to the double bond of the  $\alpha,\beta$ -unsaturated keto-grouping C-7—C-8—C-9. Hence structure XLIIb is assigned to cycloneomethynolide and structure XLIIa to neomethymycin which differs from



XLII

methymycin in that the OH group at C-10 and an atom H at C-12 are interchanged. The absolute configuration of C-4 and C-6 of neomethymycin has been established as related to L-glyceraldehyde by the isolation, from ozonolysis degradation products, of (–) methyl succinic acid which itself is related to L-glyceraldehyde. The absolute configuration of C-4 and C-6 in the macrolides methymycin, picromycin and narbomycin is probably the same, as all these compounds yield the same lactone acid XLId retaining the C-4 and C-6 of the parent compounds (221).

*Spiramycins and foromacidins.*—Three crystalline antibiotics were iso-

lated from culture fluids of *Strept. ambofaciens* and termed spiramycins I, II and III (222). They seem to be identical with the foromacidins A, B and C (32). They appear to belong to the group of macrolides. On mild acid hydrolysis mycarose (see XXXV) is liberated. On hydrolysis with 50 per cent phosphoric acid, spiramycin II liberates one mole of acetic acid, spiramycin III, one mole of propionic acid, while spiramycin I gives no volatile acid.

*Narbomycin*.—This antibiotic,  $C_{28}H_{47}O_7N$ , produced by a streptomycete, yields desosamine (39) on hydrolysis and trimethyl, 3-hydroxypimelic acid lactone on oxidative degradation (214).

#### ANTIBIOTICS OF POLYENE AND POLYENYNE NATURE

A considerable number of highly unsaturated antibiotics is now known. Some of these are polyenes, other polyenyne.

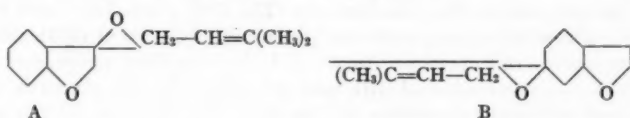
*Polyenes*.—A study (223) of the ultraviolet absorption spectra of these compounds has shown that the polyenes differ markedly from the polyenyne and arrange themselves into distinct groups. The spectra of antimycin (224), nystatin (225), rimocidin (226), and chromin (227) are very similar and resemble those of tetraenes; the absorption of flavicid (228) resembles that of a hexanene, and the absorption of candidin (229), candidin (230), trichomycin (231) and ascocin (232) that of heptadienes. The spectrum of fumagillin (233) is completely different from the rest. Fumagillin is a metabolic product of *Aspergillus fumigatus*, and possesses antibacterial and amoebicidal activity, while the others are products of actinomycetes and basidiomycetes and are characterized by high antifungal and relatively low antibacterial activity.

*Fumagillin*.—Fumagillin,  $C_{26}H_{34}O_7$  (234), is the half-ester of deca-tetra-diene-dioic acid (235) and an alcohol  $C_{16}H_{26}O_4$ ; it is split into its components by alkaline hydrolysis under nitrogen. The alcohol, termed alcohol I (236, 237) or fumagillol (228) has been obtained crystalline (239). It contains one methoxyl, one secondary hydroxyl giving rise to one atom of active hydrogen, two C-methyl and two noncarbonyl oxygen atoms (236, 237). Its analytical composition is in agreement with a structure containing one  $C=C$  double bond and three ring systems. The two oxygens form part of two of these as ether oxides. One was shown to be an epoxide ring by the thiosulphate reaction of Ross (240) and by the facts that fumagillol gave a chlorhydrin with dilute HCl and a diol on reduction, both compounds possessing two active hydrogens (238, 239). Reduction of fumagillol led to the addition of one, two or three moles of hydrogen, according to the conditions (236, 237, 239). Catalytic reduction gave a crystalline tetrahydroalcohol Ia  $C_{16}H_{30}O_4$  in which the  $C=C$  double bond and the epoxide ring were reduced and, with less active catalyst, a dihydroalcohol,  $C_{16}H_{28}O_4$ , in which only the double bond was reduced; the same product was obtained by alkaline hydrolysis of fumagillin after catalytic reduction to decahydrofumagillin. Reduction of fumagillol with lithium aluminium hydride gave a dihydroalcohol Ib  $C_{16}H_{28}O_4$  isomeric with the above mentioned dihydroalcohol, a tetrahydro-



alcohol Ib  $C_{16}H_{30}O_4$ , isomeric with the tetrahydroalcohol Iab and a tetrahydro "nor" alcohol I,  $C_{15}H_{28}O_4$ . Dihydroalcohol Ib, a diol, gave on catalytic reduction tetrahydroalcohol Iab; tetrahydroalcohol Iab was a triol in which both ether oxygens of fumagillol have been reduced to hydroxyl groups. The  $C_{15}$ - "nor" compound is also a triol; the mechanism of its formation from fumagillol is explained as a reversed aldol condensation involving the loss of formaldehyde or its equivalent. Reduction of fumagillol with sodium borohydride gave dihydroalcohol Ib and tetrahydro "nor"alcohol I, but no tetrahydroalcohol Iab. Borohydride therefore opens the epoxide ring, but not the second cyclic oxygen ether linkage.

On periodate oxidation of the diol obtained by opening of the epoxide ring in acid medium a volatile fragment is obtained probably, 4-methyl, pent-3-enal  $(CH_3)_2C=CH-CH_2-CHO$  (238). This product probably originates from the side chain of fumagillol. On ozonolysis of fumagillol a  $C_{13}$  aldehyde  $C_{13}H_{20}O_5$  is obtained (236). Further ozonolysis (238) and subsequent lithium aluminium hydride reduction of this aldehyde led to two crystalline alcohols,  $C_{11}H_{20}O_5$  and  $C_{10}H_{20}O_4$ . The formation of the  $C_{10}$  alcohol indicates loss of a formyl group during ozonolysis. The partial structures A and B for fumigillol are considered as possibilities.



The tetraene dioic part of fumagillin is rapidly destroyed by light, both in the presence and absence of air. Aerobic photolysis is accompanied by oxidation, anaerobic photolysis leads to products which are readily oxidized on subsequent exposure to air (241). The kinetics of the thermal degradation of fumagillin have been studied (242).

**Amphotericins-A and -B.**—These compounds were isolated in crystalline form by Vanderputte, Wachtel & Miller (4) from culture fluids of a streptomycete. On catalytic hydrogenation amphotericin-B absorbs 7 moles of hydrogen forming crystalline tetradecahydroamphotericin,  $C_{46}H_{87}NO_{20}$ . The nitrogen forms part of an aminodeoxy hexose  $C_6H_{13}NO_4$ , termed mycosamine, which was obtained as the crystalline tri- and tetra-acetate (243).

**Nystatin.**—The same aminodeoxy hexose mycosamine (243) was isolated from nystatin. This antibiotic, used clinically in combination with the tetracyclines to combat the secondary fungal infections frequently following application of this group of antibiotics (244, 245, 246) exerts an inhibitory effect on the aerobic and anaerobic glycolysis of yeast cells (247).

**Trichomycin.**—This antibiotic, active against yeasts and fungi, including the trichomonas group, was isolated from the culture fluid of *Streptomyces hachijoensis* (248). Purification by countercurrent distribution showed that trichomycin was a mixture containing two active substances, trichomycins A

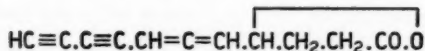
and B, accompanied by inactive impurities (249). On acetylation and oxidation with ozone acetaldehyde, glyoxal and *p*-acetamino-benzoic acid was obtained; on oxidation with permanganate of the nonacetylated sample, which gives a positive Ehrlich reaction and a positive diazo-coupling reaction, *p*-aminoacetophenone was obtained (250).

*Polyenyne*.—A review of these compounds has been published (251).

*Candidin*.—This antibiotic, produced by *Streptomyces viridoflavus*, was obtained in crystalline form. It absorbs 9 moles of hydrogen on catalytic hydrogenation, indicating that the molecule must contain 2 unsaturated groups in addition to the conjugated heptaene chromophore. The formula  $C_{46}H_{78}NO_{17}$  is in agreement with its neutralization equivalent and the analytical figures (252).

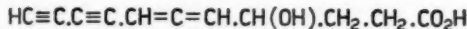
*Diatretyne 2*.—This polyacetylene antibiotic (253) produced by *Clytocibe diatrete* is the mononitrile of an octadienyne dioic acid and has the structure  $HOOC-CH=CH-C\equiv C-C\equiv C\cdot C\equiv N$ . The amide  $HOOC-CH=CH-C\equiv C-C\equiv C-CO\cdot NH_2$  which also occurs in the culture fluid of the mould is biologically inactive (254).

*Nemotin, nemotinic acid, odyssin, and odyssic acid*.—Purification of nemotin and nemotinic acid, highly unsaturated antibiotics of neutral and acidic nature produced by a basiomycete (255, 256), showed that each was a mixture of two substances, termed odyssin and odyssic acid (257). On hydrogenation of crude nemotin Anchel & Cohen obtained undecanoic acid (258) and this was confirmed with pure nemotin (259). On the basis of infrared and ultraviolet absorption spectra the lactone formula XLIIa is assigned to nemotin; nemotinic acid is the corresponding hydroxy acid XLIIb.



NEMOTIN

(a)



NEMOTINIC ACID

(b)

XLIII

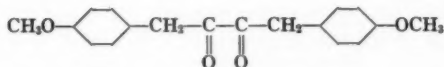
## MISCELLANEOUS

*Aureothricin and thiolutin*.—The detailed account of the studies establishing their structures as given in Binkley's review (61) has now been published (260).

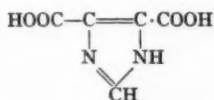
*Xanthomycin-A*.—Hydrolysis of xanthomycin-A with hydrochloric acid gave ethanolamine, ammonia, methylamine, and a large amount of humin-like material. Its instability is ascribed to the presence of a quinoid group.

Oxidative degradation of methyl tetrahydroxanthomycin, obtained with simultaneous reduction and methylation of xanthomycin, gave two crystalline products, xanthomycinic acid I  $C_7H_{11}N_3O_{10}$  and a nitrogen-free compound xanthomycinic acid II (261). An antibiotic with similar properties, possibly identical with xanthomycin-A, from the culture filtrate of a *Streptomyces* strain was isolated from Malayan soil by Dougall & Abraham (262, 263).

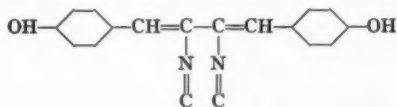
**Xanthocillin.**—Investigation of the structure of this antibiotic, produced by a strain of *Penicillium notatum*, and commercially manufactured in Eastern Germany, showed that it consisted of two components, X and Y. The major component Y has the composition  $C_{18}H_{12}O_2N_2$ . On methylation with diazomethane two methoxy groups were found. On hydrolysis of the dimethoxy xanthocillin X two moles of formic acid and ammonia were formed, indicating two nitrile groups. The rest of the molecule was identified by degradation and synthesis as 1,4 di (p-methoxyphenyl) 2,3-butadiene:



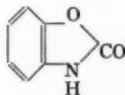
Oxidation of xanthocillin-X lead to anisic acid and imidazole-4,5-dicarboxylic acid



On the basis of these reactions (264), the structure of xanthocillin-X is thought to be 1,4-di (p-hydroxyphenyl) 2,3-diisonitrilo-butadiene



**Antifungal agents from plant seedlings.**—Virtanen and Hietala (265) have demonstrated the presence of an antifungal factor in germinated rye seedlings, identified as 2(-3)benzoxazolinone



Many other seedlings of cereal and fodder plants possess antifungal activity (266); the antifungal factor from maize and wheat seedlings was identified

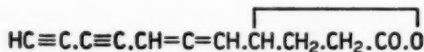
and B, accompanied by inactive impurities (249). On acetylation and oxidation with ozone acetaldehyde, glyoxal and *p*-acetamino-benzoic acid was obtained; on oxidation with permanganate of the nonacetylated sample, which gives a positive Ehrlich reaction and a positive diazo-coupling reaction, *p*-aminoacetophenone was obtained (250).

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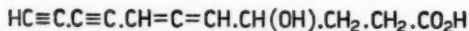
*Diatretyne 2*.—This polyacetylene antibiotic (253) produced by *Clytocibe diatrete* is the mononitrile of an octadienyne dioic acid and has the structure  $HOOC-CH=CH-C\equiv C-C\equiv C\cdot C\equiv N$ . The amide  $HOOC-CH=CH-C\equiv C-C\equiv C-CO\cdot NH_2$  which also occurs in the culture fluid of the mould is biologically inactive (254).

*Nemotin, nemotinic acid, odyssin, and odyssic acid*.—Purification of nemotin and nemotinic acid, highly unsaturated antibiotics of neutral and acidic nature produced by a basiomycete (255, 256), showed that each was a mixture of two substances, termed odyssin and odyssic acid (257). On hydrogenation of crude nemotin Anchel & Cohen obtained undecanoic acid (258) and this was confirmed with pure nemotin (259). On the basis of infrared and ultraviolet absorption spectra the lactone formula XLIIIa is assigned to nemotin; nemotinic acid is the corresponding hydroxy acid XLIIIb.



NEMOTIN

(a)



NEMOTINIC ACID

(b)

XLIII

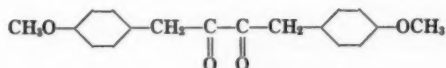
## MISCELLANEOUS

*Aureothricin and thiolutin*.—The detailed account of the studies establishing their structures as given in Binkley's review (61) has now been published (260).

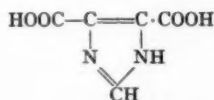
*Xanthomycin-A*.—Hydrolysis of xanthomycin-A with hydrochloric acid gave ethanolamine, ammonia, methylamine, and a large amount of humin-like material. Its instability is ascribed to the presence of a quinoid group.

Oxidative degradation of methyl tetrahydroxanthomycin, obtained with simultaneous reduction and methylation of xanthomycin, gave two crystalline products, xanthomycinic acid I  $C_7H_{11}N_3O_{10}$  and a nitrogen-free compound xanthomycinic acid II (261). An antibiotic with similar properties, possibly identical with xanthomycin-A, from the culture filtrate of a *Streptomyces* strain was isolated from Malayan soil by Dougall & Abraham (262, 263).

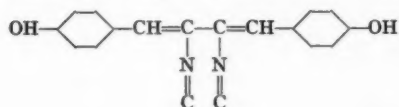
**Xanthocillin.**—Investigation of the structure of this antibiotic, produced by a strain of *Penicillium notatum*, and commercially manufactured in Eastern Germany, showed that it consisted of two components, X and Y. The major component Y has the composition  $C_{18}H_{12}O_2N_2$ . On methylation with diazomethane two methoxy groups were found. On hydrolysis of the dimethoxy xanthocillin X two moles of formic acid and ammonia were formed, indicating two nitrile groups. The rest of the molecule was identified by degradation and synthesis as 1,4 di (p-methoxyphenyl) 2,3-butadiene:



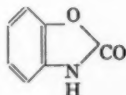
Oxidation of xanthocillin-X lead to anisic acid and imidazole-4,5-dicarboxylic acid



On the basis of these reactions (264), the structure of xanthocillin-X is thought to be 1,4-di (p-hydroxyphenyl) 2,3-diisonitrilo-butadiene



**Antifungal agents from plant seedlings.**—Virtanen and Hietala (265) have demonstrated the presence of an antifungal factor in germinated rye seedlings, identified as 2-(3)benzoxazolinone



Many other seedlings of cereal and fodder plants possess antifungal activity (266); the antifungal factor from maize and wheat seedlings was identified

as 6-methoxy 2(-3) benzoxazolinone (267) and synthesized (268). For a review of this work see (269).

Reviews on various aspects of antibiotics research have appeared in the *First European Symposium on Antibiotics* (270).

Several antibiotics have recently been described in Japanese publications; most of these have as yet not been sufficiently characterized to allow comparison with the known antibiotics. They are listed as follows:

A-6 substance (271), Allomycin (272), Amaromycin (273), Antiblastin (274) Blasticidin (275), Caliomycin (276), Carzinocidin (277), Cereviocidin (278), Eurocidin (279), Gancidin (280), Grisamine (281), Homomycin (282), Mesenterin (283), Mikamycin (284), Mitomycin (285), Monilin (286), Phleomycin (287), Pluramycin (288), Pyridomycin (289), Ractinomycin (290), Roseothricin (291), Sinanomycin (292), Tertiomycin-A (293), Tertiomycin-B (294), Thiomycin (295), Toyocamycin (296), Violacetin (297).

I wish to thank Dr. Marcella Magliola for her invaluable help in the preparation of the bibliography of this review.

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## BIOCHEMISTRY OF FISHES<sup>1,2</sup>

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Since this subject was last reviewed (1) articles summarizing the advances which have been made in various phases of the biochemistry of fish have appeared from time to time. Recent publications have dealt with physiology (2), marine biochromes (2, 3), assessment of nutritive value and analytical data (2, 4 to 7), and marine oil research (6 to 10). These general topics have therefore been excluded from the present review, which has been devoted to a limited number of general subjects of fairly immediate interest. With the exception of the section on marine sterols, a subject in which research has been almost exclusively confined to invertebrates, only literature dealing with true fish (Pisces) has been considered.

### PROTEINS

A number of important contributions to our knowledge of fish muscle proteins have been made. The general fractionation methods employed (11 to 15) have resulted in better separation of the proteins than was obtained by earlier investigators (16, 17, 18), largely because of the availability of the techniques of electrophoresis and ultracentrifugation.

The proteins extractable by cold neutral salt solutions of fairly high ionic strength include myosin, actin, and tropomyosin. The conditions for extraction employed by different investigators have not been consistent so that the results are not strictly comparable; post-mortem age, degree of comminution, pH, extraction time, and ionic strength of extractant solution may all have affected results. Salt (e.g., NaCl, KCl, LiCl) solutions of  $\mu$  0.5 to 1.0 and pH 6.0, and preferably between pH 7.0 and 7.5, have usually proved satisfactory (11, 13, 14) for extraction. Solutions of  $\mu$  0.3 to 0.45 have proven successful with some muscles (12, 15). Most investigators who have studied the protein fraction extractable under the above conditions, which is precipitated by adequate dilution with water ( $\mu < 0.25$ ), have

<sup>1</sup> The survey of the literature pertaining to this review was completed in October, 1957.

<sup>2</sup> The following abbreviations are used in this chapter: ADP for adenosine diphosphate; AMP for adenosine monophosphate; ATP for adenosine triphosphate; ATPase for adenosine triphosphatase; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; DR-1-P for deoxyribose-1-phosphate; GDP for guanosine diphosphate; GMP for guanosine monophosphate; GTP for guanosine triphosphate; IMP for inosine monophosphate; PAB for *p*-aminobenzoic acid; PGA for pteroylglutamic acid; RNA for ribonucleic acid; UDP for uridine diphosphate; UMP for uridine monophosphate; and UTP for uridine triphosphate.

<sup>3</sup> The assistance of Dr. D. R. Idler in preparing the section on Sterols of Marine Invertebrates is greatly appreciated.

worked with more or less pure actomyosin. The ultracentrifuge pattern of carp actomyosin extracted at pH 7.0 showed a single sharp peak, and in its physical properties it did not differ in some respects from rabbit muscle actomyosin (11, 12). Data on the physical properties of actomyosin prepared from other fishes are lacking.

Myosins were prepared from carp (11) and from cod (19) muscles by different procedures, but were not homogeneous by ultracentrifugal analyses, probably due to the presence of contaminating components. When carp muscle actomyosin was subjected to ultracentrifugal analysis in the presence of ATP, myosin, with a sedimentation constant of 6.55, which is not greatly different from that of rabbit myosin, separated (11, 12). It has been found that the amount and kinds of stroma proteins in both cod and carp muscles make it more difficult to prevent simultaneous extraction of actin and myosin (as actomyosin) than is the case with rabbit muscles. There has also been found to be a parallel between the rate of extraction of actin from minced fish muscles and the amount of stroma proteins present (20).

Tropomyosins have been prepared from fish muscles by solvent extraction (12, 21, 22), and by ammonium sulfate fractionation and electrophoresis (22). Several tropomyosins with somewhat different physical properties, including crystalline tropomyosin and nucleotropomyosin, have been prepared from carp muscles and their physical properties described (11, 12, 21, 22, 23). G actin has been prepared from cod muscle, and in the presence of salts this polymerized to viscous F actin (19). F actin has also been demonstrated in carp muscles (11).

Recent investigations have shown that the rate at which myosin in intact frozen cod muscles denatures during prolonged frozen storage (e.g., 390 days at  $-30^{\circ}\text{C}.$ ) is influenced markedly by the rate at which the tissues have been frozen. There appears to be a rather critical freezing period above or below which myosin denaturation, as measured by its tendency to become insoluble in cold neutral 0.85 *M* NaCl solutions, is comparatively slight (24). It has long been considered that the organoleptically detectable toughness of fish muscles which occurs on prolonged frozen storage, particularly at elevated temperatures (e.g.,  $10^{\circ}\text{C}.$ ), is directly connected with denaturation of myosin as measured by its insolubility in neutral salt solutions of high ionic strength (25). However, recent investigations have shown that, under certain conditions, the former alteration occurs with only an extremely slight decrease in myosin solubility (24, 26).

The separation of fish muscle myocommata from the myotomes offers considerable promise in facilitating determinations of the myosin content of the latter, and the effect of age, sexual development and season on this (27). Changes in viscosity and sulfhydryl groups in stored frozen fish muscles have been measured (28).

Fish muscle proteins extractable by salt solutions of low ionic strength ( $\mu < 0.15$ ) include those which have been named myogens, myoalbumins and globulin X. They normally account for about 21 to 22 per cent of the total

muscle protein of teleost fishes (11 to 14, 29, 30). The low ionic strength extractable protein fraction obtained from a number of marine and fresh-water fish, including teleosts and elasmobranchs, has been subjected to electrophoresis both by the moving boundary and zone methods (11, 12, 15, 31, 32, 33, 34, 35). There appear to be from seven to ten different protein fractions in these extracts as determined by the above methods, and, where comparisons have been made, both methods yield somewhat similar results. It seems to be possible to divide these proteins into three main groups, and there is a fairly general similarity in the electrophoretic patterns obtained, but there are distinct species differences. Scanty available data do not indicate that there is any important difference between the electrophoretic patterns of low ionic strength extracts of carp white and red lateral muscles (12). A crystalline myogen with a molecular weight of about 67,000 has been isolated from carp muscles, and it appeared to be homogeneous by ultracentrifugal analysis (36), but gave two peaks by electrophoresis (37). The electrophoretic inhomogeneity has been shown to be due to a small amount of a contaminating protein with a comparatively low molecular weight. A very similar protein, but one with a different sedimentation constant, has been isolated from flounder muscles (38).

Red lateral muscles of certain fish have been shown to be rich in both cytochrome and myoglobin (39), and crystalline myoglobins have been isolated from several species of fish (40, 41, 42). Red lateral muscles of fish have been reported as being rich in cytochrome-*c* (39), and cytochrome-*c* isolated from tuna red muscle has been reported to differ somewhat spectroscopically from that of horse heart muscle (43). Crystalline cytochrome-*c* preparations isolated from the heart muscles of tuna species, and from bonito, appear to be practically identical spectroscopically, but differ somewhat from that of crystalline beef heart muscle cytochrome-*c* (44). The haemoglobin content of the blood of migratory fish has been reported to be greater than that of less active fish (45). The haemoglobin content of fish blood is very variable, e.g., 2.0 to 11.8 gm. per 100 ml. (46, 47). However, this is to be expected in view of the findings that the amount of respiratory pigment and erythrocytes depends on the oxygen requirements of fish (48).

A number of different methods of preparation of the protamines have been recorded. These preparations have not been absolutely homogeneous when examined by the techniques of electrophoresis, ultracentrifugation or counter current distribution (50, 51, 52). Clupein, salmine, and sturine are of relatively simple composition and contain six to 10 different amino acids. Molecular weights of from 3,800 to 10,000 have been reported for different protamine preparations (51 to 54). The arginine content of clupein has recently been reported as about 88 per cent (49). Recent physical and chemical investigations with various protamines indicate that they are composed of a number of different proteins of different molecular weights and amino acid composition (51, 53, 54). Considerable progress has been made in determining the N- and C- terminal amino acids in certain of the protamines, and

also the amino acid sequence (51 to 57). It has been indicated that about one mole of nucleic acid combines with 100 of protamine, and that a large number of different nucleoprotamines could exist (51).

The only connective tissue protein of fish which has been studied to any extent is collagen, and much of the early information regarding this has been reviewed (58). X-ray studies have indicated that teleost collagen, ichthyocol, differs somewhat from the elasmobranch collagen, elastoidin. Many differences have been found between the collagen obtained from codfish skins and bovine collagen (59, 60). The total amino acid composition of a number of fish collagens has been determined, and a very similar distribution found. However, there were notable differences, especially with respect to a lower hydroxyproline and proline content, from mammalian collagen (61). About one third of the total nitrogen of whole herring appears to exist as gelatin nitrogen, which is divisible into three fractions as based on solubility criteria. Significant seasonal changes in the proportions of these fractions have been found (62). The essential amino acid composition of whole herring scales has been determined (63), and the following composition of pilchard scales has been reported: mineral matter, 56 per cent; ichthylepidin, 21 per cent (molecular weight about 200,000); and gelatin, 23 per cent (64). A pseudokeratin, designated ichthulokeratin, has been found in salmon eggs. It has a histidine:lysine:arginine ratio of 1:3:4.5, and is digested by pepsin and not by trypsin (65). The amino acids occurring in acid hydrolysates of fish lens proteins have been reported (66). The rather limited number of studies which have been carried out on fish plasma proteins indicate that there are great variations between both closely related and unrelated species, and that there is little or no protein which is similar electrophoretically to the gamma globulin of the mammals (32, 67, 68, 69).

#### NONPROTEIN NITROGEN

This fraction is said to account for from 9.2 to 18.3 per cent of the total nitrogen in teleost fishes and from 33 to 38.6 per cent in elasmobranch fishes. The compounds occurring in this fraction have been grouped as follows: (a) volatile bases (ammonia, mono- di- and trimethylamine), a group which is of minor significance in muscles of living fish; (b) trimethylammonium bases (trimethylamineoxide, betaines); (c) guanidine derivatives (creatine and arginine); (d) imidazole or glyoxaline derivatives (histidine, carnosine, and anserine); (e) miscellaneous (urea, amino acids, and purine derivatives) (70). The occurrence of these soluble nitrogenous compounds in the muscles of fish has been the subject of fairly extensive investigation.

An alcohol extract of haddock muscle was subjected to displacement chromatography and the amino acids and related compounds in the various fractions were identified by paper chromatography. The following substances were found: leucine, glycine, alanine, glutamic acid, serine, creatine, creatinine,  $\beta$ -alanine, trimethylamineoxide, anserine (comparatively large amounts), valine, threonine, choline, aspartic acid, cysteine, histidine,

methylhistidine, lysine, arginine (moderate amounts), and traces of tyrosine and phenylalanine, Ammonia, mono- and dimethylamines were present in only trace amounts and carnosine was absent. Crystalline anserine was isolated and identified (71). Subsequent investigations of nine species of gadoid fish showed that the content of soluble nitrogenous compounds in the muscle was rather similar to that of the haddock. However, some interesting differences were noted. Thus, betaine and sarcosine were present only in elasmobranch fish, and anserine and methylhistidine in the gadoid fish. Traces of glutathione were found. Crystalline betaine was prepared from skate muscle (72). Further work (73) showed that flat fish yielded smaller amounts of trimethylamineoxide and anserine than did gadoid fish, and that 1-methylhistidine appeared to be absent. Pelagic fish (tuna and mackerel) were unique in that their muscles contained comparatively large amounts of histidine but no anserine or carnosine. Several unidentified compounds were demonstrated in the above studies. This work indicated that there is probably a definite generic pattern in the distribution of certain of the soluble nitrogenous compounds in fish muscles.

Taurine, which was not detected by the above investigators due to the ion exchange resin technique employed, was subsequently demonstrated in comparatively high concentrations in extracts of cod muscle. It was closely similar in concentration to trimethylamineoxide and creatine which occur in fish muscles (74). The following concentrations of free amino acids (mg. per 100 gm. wet weight) have been reported in cod muscles (75 per cent ethanol extracts used): taurine 300, glycine 21, alanine 15, glutamic acid 8, 1-methylhistidine 6.7, leucine 7.1, valine 3.5,  $\beta$ -alanine 1.2, aspartic acid 1.9, serine 1.4, lysine 1.9, cysteic acid 1.2, histidine less than 0.5, and traces of proline, tyrosine, phenylalanine, and threonine. Interesting seasonal variations in the concentrations of certain of these extractives were observed. Thus taurine reached a peak in May to July (300 to 450 mg.) and was at a minimum in December to February (100 to 150 mg.). The lysine content, which was 0.5 mg. throughout most of the year, rose to 12 to 15 mg. in January and February during the spawning period (75). Recently an increase in the lysine content of both male and female sturgeon muscle during spawning migration has been reported (76). It has been shown that, with few exceptions, the free amino acid content of aseptically excised fish muscles does not increase appreciably in 25 days at 0°C. The glutamic acid content increased from 10 to 29 mg. per 100 gm., the  $\beta$ -alanine from 2.5 to 66 mg. per 100 gm. and the 1-methylhistidine content from 6.2 to 102 mg. per 100 gm. Comparatively slight decreases in the alanine and lysine content were observed (77).

The distribution of the imidazole bases histidine, 1-methylhistidine, carnosine, and anserine, in the flesh of a large number of North Pacific fish marine mammals, and invertebrates, has been studied. Quantities of these bases varied from approximately 2 to 56.8  $\mu$ g. per gm. Interesting species differences in the distribution were recorded (78). Paper electrophoresis of



aqueous extracts of fresh crawfish and mussels have produced fraction samples which are specific for each species, and the electrophoretic patterns change during prolonged storage up to 40 days at 0°C. (79). It has been reported that the muscles of crustacea contain a greater content of free amino acids than do those of the fishes (80). A peptide was isolated from the muscles of a number of sea fish that did not occur in the muscles of fresh-water fish. This peptide, on acid hydrolysis, yielded glutamic acid, aspartic acid, glycine or serine,  $\alpha$ -alanine and very little threonine (81).

The distribution of urea, trimethylamineoxide, and ammonia in a large number of species of fish is now known (70, 82). It has been suggested that the desirable flavour of a number of different molluscs and fishes is due to the high content of free amino acids and more particularly to the mono-amino acids (82 to 85), and in case of certain pelagic fish, to the histidine (86) present in their muscles.

#### NUCLEIC ACIDS AND RELATED COMPOUNDS

The discovery of the carp muscle protein, nucleotropomyosin, marked the first clear-cut demonstration of the presence of RNA in fish muscles (22). More recently it has been demonstrated that RNA is present in concentrations of from 42–142  $\mu$ g. per gm. in the muscles of Pacific Ocean fish (87). RNA has been prepared in comparatively pure state from muscles of lingcod and sockeye salmon (87), and the distribution of the bases adenine, guanine, cytosine and uracil (molar ratio approximately 1:2:1:1.25) has been found to differ somewhat from that of the RNA of carp nucleotropomyosin (molar ratio 1:2.1:1.85:1.10) (22). The ratio of purines to pyrimidines was somewhat higher, and of nitrogen to phosphorus somewhat lower, than that recorded for yeast RNA (87). The enzymic degradation of RNA by fish muscle enzymes is being studied actively (see section on Enzymes).

Initial attempts to determine DNA in fish muscle aqueous extracts met with failure (22, 88), probably due to the comparatively low concentrations in which it occurs, and the fact that cell damage is apparently required to liberate it from the muscle cells (89). The highest concentration of DNA phosphorus obtained from cod muscle press juice was 0.628 mg. per 100 ml. (90). The DNA content of the expressible fluids of fish muscles appears to yield useful information regarding cell damage which occurs during freezing (90, 91). The muscles of six species of Pacific coast fish contained from approximately .02 to 0.25 mg. of DNA phosphorus per 100 gm. of wet muscle (87). These values are considerably lower than those recorded for mammalian muscles where concentrations from 4.3 to 9.6 mg. of DNA phosphorus per 100 gm. have been reported (92). Muscles of Pacific herring have been found to contain between 1.87 and 3.51 mg. of DNA per 100 gm., and between 10.4 and 26.5 mg. per 100 gm. of RNA (93). The following molar ratio of bases in highly polymerized herring roe DNA has been recorded: adenine 1.0, guanine 0.55, cytosine 0.70, thymine 1.01. Chemical degradation studies of this product suggested that there are regions where at least three pyrimidine nucleo-

tides are linked together (94). It has been reported that a single whole or cytolized defatted trout sperm contains  $2.45 \times 10^{-9}$  mg. of DNA and  $1.55 \times 10^{-9}$  mg. of arginine (95).

Several investigators have studied the nucleotides of fish tissues, and more particularly of the muscle. An investigation of the muscles of five Pacific coast fish which were obtained in considerably different physiological condition indicated that the sum of the ATP and ADP in these muscles varies from 0.27 to 3.72  $\mu$ M per gm. expressed as ATP. The lower values were obtained particularly in fish muscles which were stored for two days at 0°C., or had been obtained from fish which had struggled considerably (96). Rather similar values have been obtained with pike and tench (97). The nucleotide content of skeletal muscle of the cod has been investigated in detail (98). For rested fish the following values were obtained ( $\mu$ M per gm.): ATP, 5.34; ADP, 0.576; AMP, 0.069; DPN, 0.107; IMP, 1.26. Decreases in both ATP and ADP were noted in exhausted fish or in fish which had just passed out of rigor. In this study comparatively large amounts of IMP accumulated post mortem due to the activity of a deaminase. Small amounts of GTP, UTP, CTP and "ADX" were identified among a number of minor constituents. The presence of a nucleotide which contains a purine substituted in the C-6 position, and which has an absorption spectrum similar to that of adenylysuccinic acid, has been demonstrated in cod livers (99). Salmon livers have been found to contain DPN, AMP, GMP, UMP, IMP, ADP, UDP, GDP, ATP, uric acid, ADP-acetylglucosamine (100), and a new adenylysuccinic acid derivative containing sulfate and a peptide (101).

It has been stated that the RNA content of salmon eggs is greatest during embryonic growth and that during critical periods the RNA values are low (102). It has also been reported that in the development of ovogonia to ovocytes the RNA in the cytoplasm increases and declines after the appearance of yolk granules (103). Ripe salmon testes contain 7.5 per cent of sodium deoxyribonucleate and a method whereby this has been isolated almost quantitatively has been described. Salmine can be prepared readily as a by-product of this method (104).

#### PHOSPHOLIPIDES

The literature concerning the very plentiful triglycerides of fish tissues has been much more extensive than that relating to the much less plentiful phospholipides. The content in fish tissues of phospholipides is comparatively low as the following values indicate (per cent of wet weight): flesh 0.1 to 0.85; (105 to 109); liver 0.16 to 0.49 (106, 110, 111); eggs 0.4 to 4.0 (114, 115); intestines 0.41 to 1.0 (106, 112); brains about 4 per cent (116, 117, 118), and spinal cord about 6 per cent (119).

Until recently the subdivision of the total phospholipide fraction of fish tissues has proved difficult due to the inadequacy of the fractionation procedures available. Thus attempts made prior to about 1952 to divide the phospholipides of fish organs into fractions such as lecithin, cephalin and

sphingomyelin have yielded results which can only be regarded as rough approximations (120). Practically all recent work on fish muscle phospholipides has been carried out with cod and haddock in which the total lipide of the flesh is relatively low and practically free of triglycerides. Thus both cod and haddock muscle contain approximately the same amount of total lipides, namely 0.6 per cent. The composition of this fraction in haddock and cod flesh respectively has been recorded as follows (per cent wet weight): lecithin 42.8, 35; unidentified lipides 15.2, 21; waxes and alcohols 10.5, 13; free cholesterol 6.1, 8; free fatty acids 6.1, 6.0; phosphatidyl ethanolamine 5.4, 8.0; inositol lipides 4.4, 2.0; cholesterol esters 3.4, 5.0; triglycerides 2.4, 3.0; hydrocarbons demonstrated in the earlier work are probably contaminants (108, 121). Quantitative extraction has involved use of a series of solvents (122), removal of impurities from the extract so obtained (108, 109), and fractionation of the purified extracts by means of counter-current distribution between aqueous ethanol and light petroleum. Resulting fractions, or hydrolysates obtained from them, have been analyzed for all known constituents. The sum of the content of fatty acids, total unsaponifiable matter, free and esterified cholesterol, higher aliphatic aldehydes, glycerophosphoric acid, glycerol, inositol, choline, ethanolamine, and serine has been used to indicate the composition of a particular fraction, and thus of its total composition. The results have indicated the great complexity of the mixture of lipides in these tissues, and also the presence of new and uncharacterized lipides. The following values have been reported for the phospholipides of the flesh of the cod and haddock respectively (percentage of total phospholipides): lecithin 54, 62; phosphatidyl ethanolamine 11, 8; phosphatidyl serine, absent; inositol phosphatides 3, 6; plasmalogens, present, 2; novel phospholipides 32, 22 (120).

A fairly extensive study has been made of the novel phospholipide fractions. Traces of sphingosine nitrogen were found in certain of the fractions. However, sphingomyelin was never positively demonstrated, though its presence in trace amounts could not be completely ruled out. Novel phospholipides of haddock flesh were arbitrarily divided into three components, namely A, B and C, which occurred in the approximate ratio of 7:1.5:1.5. Lipides A and C, which were not obtained pure, had ratios of fatty acid to glycerol and phosphorus in excess of those usually found in esters of this type. They appeared to be esters of a polyglycerol phosphate in which the fatty acid: glycerol:phosphorus ratio was 4:2:1 (108, 123), corresponding to a *bis*-phosphatidic acid. However, neither of them possessed acidic properties. Fraction C, which was difficult to extract, was not identified. These three fractions also occurred in cod flesh, but in different proportions. Fractionation of the richest concentrates of inositol lipides from cod and haddock muscle indicated that the ratio of glycerol to inositol in these was approximately 2:1. They were very resistant to extraction, were present in considerably different proportions in cod and haddock muscles, and more than one type of compound was found (108, 120). Phosphatides

of serine, 2-aminoethanol and inositol have been demonstrated in the brain cephalins of Pacific pollock (125). It appears that the eggs of the same fish contain a serine phosphatide in relatively high concentration (126).

The distribution of C16, C18 and C20, saturated fatty acids, and C18, C20 and C22 unsaturated fatty acids, and the degree of unsaturation of the latter, have been reported for the following phospholipid fractions from haddock flesh: acetone-soluble lecithins, acetone-insoluble lecithins, total lecithins, and lipid fractions A, B, and C (120). The major fatty acid component of haddock depot fat, namely hexadecanoic acid, was virtually absent from all haddock lipid fractions (124). The lecithins had a remarkably high content of C20 and C22 unsaturated fatty acids, the acetone soluble lecithins having in general a greater degree of unsaturation than the acetone insoluble lecithins. The acetone-soluble lipid fraction of the eggs of a shark has been found to contain C14, C16, C18, and C20 saturated fatty acids, and C16, C18, C20, C22 and C24 mono- or polyunsaturated fatty acids. Cholesterol, chymyl and batyl alcohols were isolated from the unsaponifiable fraction (127). The egg lecithins of pollacks yielded C14, C16, and C18 saturated fatty acids, and C16, C18, C20, and C22 unsaturated fatty acids (128).

#### ENZYMES

Seasonal variations in proteolytic enzyme activity of skipjack tuna digestive tracts have been observed (129). Crystalline pepsins have been prepared from the stomachs of salmon (130) and three species of tuna (131). These differed from hog or beef pepsins in substrate specificity, crystal structure and amino acid composition. The presence of a cathepsin has been demonstrated in the stomachs of certain fresh-water teleost fish (132), while the gastric mucosa of an elasmobranch fish has been found to possess both a cathepsin and a pepsin with pH-activity optima of 2.0 and 4.5, respectively (133).

Post-mortem proteolytic activity in fish tissues has been investigated to a limited extent, bacterial growth in whole fish muscle during prolonged holding periods being inhibited by chemical preservatives (e.g., toluene), or by prior sterilization of the tissues by ionizing radiations. With one exception (134), slow proteolysis has been observed as judged by increases in tyrosine-, ammonia- or amino-N (97, 135, 136). A recent investigation in which extracts of tissue homogenates were used for determining "cathepsin" (pH 4.0) and "neutral protease" (pH 7.6) activity, showed that the latter was absent in muscles or organs of several widely different species of fish. On the other hand the muscles, and more especially the liver and spleen, possessed much more marked cathepsin activity than did similar mammalian tissues (137).

An anserinase enzyme (pH optimum 6.3-7.5) has been demonstrated in cod muscles and partially purified (138, 139). These muscles also possess another dipeptidase (pH optimum 3-4) which hydrolyzes carnosine and

certain other dipeptides (140). A glycylglycine dipeptidase has been found in fish muscles (137). Further investigation concerning the occurrence, specificity, and properties of these peptidases is needed.

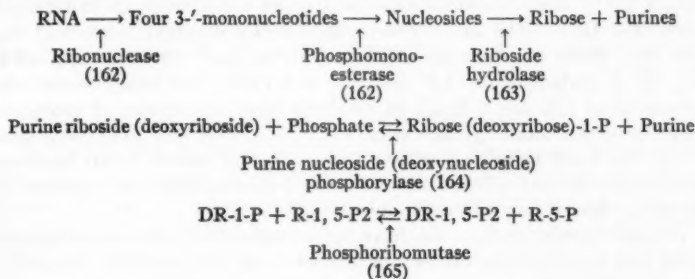
Arginase has been reported to occur in red lateral muscles of tuna (141) and in dogfish, but not in skate muscles (142). Amino acid decarboxylases have been variously reported as being probably absent (137) and present (143) in fish muscles. Catalase has been found in red lateral muscles, white muscles and livers of fish, and its seasonal distribution and some of its properties investigated (144). Carbonic anhydrase activity has been demonstrated in tissues of teleost fishes (145), and is said to play an important part in brachial excretion of bicarbonate in dogfish (146). Trimethylamine-oxide reduction, long thought to be associated solely with cells of bacteria occasioning post-mortem fish spoilage (147) has now been shown to occur in sterile fish muscles (134, 148, 149). Trimethylamineoxide is readily reduced at 20–22°C. in the presence of certain nonenzymic catalysts (150) which are almost certainly present in fish muscles, and especially in their red lateral muscles, so that the phenomenon may be nonenzymic in origin.

The glycogen content of fish muscles varies with the season, decreases markedly when fish struggle prior to capture, and the quantity present has reportedly never exceeded 850 mg. per 100 gm. (96, 151 to 154). Post-mortem glycogenolysis results in the formation of lactic acid, which is more rapid at 15°C. than at 0°C., but which, in the temperature range 0° to –10°C. is maximal at about –3°C. (153, 154). Free reducing sugar (152), recently identified as glucose, occurs in fish muscles in concentrations which range from 1 to 74 mg. per 100 gm. (155, 156). There is some confusion regarding the origin and fate of glucose in fish muscles post mortem. Thus it has been found that, in certain fish frozen in liquid nitrogen, the muscle glucose content is negligible, and that it increases markedly after a few days at 0° (156). On the other hand, it has been stated that glucose is normally present in cod muscles at time of slaughter, and decreases very slowly at 0° due to bacterial action (155). In fish liver both lactic acid and reducing sugar accumulates post mortem (157). The enzymic steps which are involved in the above degradations remain to be determined.

Fish muscles possess marked ATPase activity which, especially at 0°C., is considerably greater than that of warm-blooded animals (11, 97, 158). Two actomyosin fractions having ATPase activity with optima at pH 9 and at pH 6.5 or 7.0 have been isolated from carp muscles and certain of their properties studied (159). A decrease in ATP, an increase in orthophosphate, and formation of inosine, AMP and  $\text{NH}_3$  were reported in muscles of frigate mackerel which had struggled prior to slaughter (160). This indicated that these muscles probably possess an ATPase, ADP deaminase and a phosphomonoesterase. No appreciable decrease in ATPase activity has been demonstrated in fish muscles stored at –18°, –76° and –190°C. for several months (161).

Many of the enzymic steps leading to the post-mortem formation of

ribose and pentose phosphate esters in Pacific lingcod muscles have been demonstrated as indicated below, and a number of the enzymes concerned partially purified.



The phosphoribomutase enzyme effected an equilibrium between R-1-P, R-5-P, DR-1-P, DR-5-P, R-1,5-P<sub>2</sub> and DR-1,5-P<sub>2</sub>. Guanosine, adenosine (adenine) deaminase (166) and phosphodiesterase enzymes have been demonstrated in lingcod muscles (167). The comparative importance of RNA and ATP as initial substrates for the post-mortem enzymic formation of ribose in fish muscles remains to be determined, though the possible steps leading to formation of this pentose from ATP in cod muscles have been outlined (168). Free ribose accounts for much of the Maillard type of browning which may occur in heated or dried fish muscles (169, 170), and enzymically formed 1-methylhistidine may also be involved to some extent (171, 172). Alkaline phosphatase has been found in the gills (173) and kidneys (174, 175) of certain fish.

Some of the enzyme systems of fish tissues which are involved in intermediary metabolism have been investigated. The addition of pyruvic or oxalacetic acids to certain fish muscles has been found to support formation of citric acid, which occurs in concentrations of between 2.6 and 50 mg. per 100 gm. (176). A malonic acid decarboxylase has been found in muscles of carp and other fish (177). In muscles and livers of carp and minnows evidence has been accumulated which indicates that many of the enzymes concerned in the citric acid cycle, in glycolysis (e.g., glucose-6-phosphate and 6-phosphogluconate dehydrogenases), and in oxidation of fatty acids (mitochondrial enzymes) are present (178).  $Q_{10}$  values have been determined for tissue slices from adult coho salmon using different substrates in a Krebs's medium II (179), and the following decreasing order of activity found: brain, heart, liver, kidney, red lateral muscle, and ordinary skeletal muscle (180).

#### NUTRITION

The environment in which fish live has made a study of the nutrition somewhat more complicated than is the case with most warm-blooded experimental animals. Some of the conditions which should be observed for



proper care of hatchery fish during nutrition experiments have been outlined (181). It has been stated that the protein requirement of fish is high, and the carbohydrate requirement low, in comparison with warm-blooded animals (182). High fat diets, especially those containing rancid fats, are undesirable (183). The mean composition of 69 different salmonoid fish production diets has been recorded as follows (per cent): protein, 62.4; lipide, 23.0; carbohydrate, 4.8, and ash, 10.0 (184). The exact mineral requirements of fish are difficult to establish since absorption of inorganic ions from the surrounding water occurs (183, 185, 186). The only recognized mineral deficiency in fish arises from lack of dietary iodine, but it has been assumed that fish probably require all mineral elements that are necessary to support the life of warm-blooded animals (183).

Vitamin requirements of fish have been studied by a number of investigators, but much of the earlier literature is confusing and contradictory. Young trout do not appear to require either vitamin A or D for normal growth (187, 188). The first significant work concerning the requirements of fish for vitamins of the "B complex" was carried out using a synthetic control diet, and it was found that rainbow trout required pantothenic acid, PGA, and inositol but not vitamin B12,  $\alpha$ -tocopherol, biotin, nicotinic acid, ascorbic acid or vitamin K (189). Using this basal diet it was subsequently reported that brook trout (190) and brown trout (191) require choline, nicotinic acid, PGA, inositol, pyridoxine, and riboflavin. They also appeared to require biotin (191), but not vitamin B12 or PAB (190, 191). Recent work, in which chinook salmon fry were fed a synthetic ration which contained vitamin free casein, tryptophan, methionine, purified corn oil, minerals and vitamins, has shown that withdrawal of certain of the vitamins from the ration causes deficiency symptoms. Under the experimental conditions a definite requirement for thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, inositol, biotin, PGA and choline, but not for ascorbic acid, PAB or vitamin B12 was demonstrated (192).

The first report concerning the amino acid requirements of fish has appeared recently. Using a synthetic diet (193) it was found that chinook salmon fingerlings required nine "essential" amino acids, namely, arginine, histidine, lysine, leucine, methionine, phenylalanine, threonine, tryptophan and valine. No requirement for alanine, aspartic acid, cystine, glycine, glutamic acid, proline, serine and tyrosine was demonstrated (194). The utilization of carbohydrates orally administered to brook trout has been investigated. Absorption rates, blood sugar, liver glycogen and muscle glycogen were determined, and the danger of feeding high levels of carbohydrates to fish, which usually results in high glycogen concentrations in the livers due to the inability of fish to excrete carbohydrate, was stressed (195). It has long been known that fish absorb sugars from the surrounding water, and recent studies have dealt specifically with absorption of glucose (196), glucose and sucrose (197), and glucose, galactose and pentoses (198).

The absorption or ingestion (199 to 204), distribution (205 to 209),



and elimination (204, 208) of inorganic ions in fish has been studied extensively. Many of the investigators have used radioactive tracer techniques, and some consideration has been given to "tagging" fish in this way (210, 211). The many attempts to increase growth of young fish by inclusion of antibiotics in their diets have resulted in either no stimulation or a slight depression of growth (212).

#### STEROLS OF MARINE INVERTEBRATES

There have been two reviews on studies of the sterols of marine invertebrates prior to 1951 (213, 214), so that the present discussion has been devoted primarily to advances since that time. Particular emphasis has been placed on Japanese studies because as yet much of this work is unavailable in English. Much of the Japanese effort has been directed to a screening program involving a large number of classes of marine invertebrates, and the results are of a preliminary nature (215 to 221).

*Mollusca*.—Two species of chiton (*Amphineura*) have been shown to contain  $\Delta^7$ -cholestenol (222, 223). The sterol of one fresh-water snail (*Gastropoda*) is reported to be  $\beta$ -sitosterol (224), while another is said to contain brassicasterol (7-dihydroergosterol), poriferasterol (24,  $\alpha$ -stigmasterol), cholesterol and  $\gamma$ -sitosterol (225). Two other *Gastropoda* are reported to contain cholesterol (226).

In few fields has the use of trivial names caused as much confusion as have those given to unhomogeneous or poorly characterized marine sterols. *Ostreasterol* has been claimed to be identical with a sterol isolated from a sponge and named *chalinasterol* (213, 214, 227). *Chalinasterol*, in turn, was supposedly shown by ozonolysis to be 22-dehydrocampesterol (228), and on the basis of physical properties the sterol of two sea anemones was said to be identical with *chalinasterol* (229). The sterols of the oyster and other bivalves were shown to be complex mixtures from which a pure sterol could not be isolated by a few simple crystallizations (230, 231). Employing chromatography, 24-methylencholesterol was shown to be a major sterol component of several bivalves and this structure was confirmed by partial synthesis (230, 231, 232). Other data support the conclusion that *ostreasterol* is a mixture of 24-methylencholesterol and other di-unsaturated sterols (233). The sterol from one of the sea anemones, originally reported to be *chalinasterol*, has now also been shown to be 24-methylencholesterol and apparently contains none of the sterol which gave methylisopropylacetaldehyde upon ozonolysis and on which the original structure of *chalinasterol* was based (234). Therefore, it has been demonstrated that *chalinasterol*, named for the sponge from which it originated, is not a chemical entity.

The presence of  $\Delta^{5,7,22,25}$ -cholestatetraenol (235) has been demonstrated in the sterols of a clam. A sterol isolated from several species of clam and named *corbisterol* has been shown to be 7-dehydrostigmasterol (236 to 241). However, brassicasterol, poriferasterol,  $\gamma$ -sitosterol and cholesterol have been indicated to be components of bivalve sterols (226, 242, 243). Since none of

the investigators was aware of the presence of 24-methylencholesterol (230, 231) in Pelecypoda, and since most identifications have been based primarily on physical properties, it would seem undesirable to attempt to draw conclusions concerning the remaining sterols of bivalves at the present time. It is known, for example, that the sterols of at least one species of mussel and clam gave isopropylacetaldehyde and methylisopropylacetaldehyde, and no ethylisopropylacetaldehyde upon ozonolysis which indicates the presence of a  $\Delta^2$ -sterol in the cholesterol series but no poriferasterol (244).

*Echinodermata*.—The comparative biochemistry of the sterols and other components of Echinodermata has received considerable attention (213, 245). It is of interest that one species of Ophiuroidea is reported to contain  $\beta$ -sitosterol (246) and another species to contain  $\beta$ -sitosterol, poriferasterol,  $\gamma$ -sitosterol and stigmasterol (247) rather than cholesterol (213). Thus Echinoidea are the only Echinodermata known to contain cholesterol, and this has been confirmed for another sea urchin (248). The sterols of starfish (Asteroidea) have long been known to be of the 7-unsaturated type. Delta 7-cholestenol, previously found in mammalian skin (249) has been isolated from a starfish (250, 251, 252). Hitodesterol isolated from starfish sterols has been shown to be identical with  $\alpha$ -spinasterol (7,22-stigmastadienol) (253, 254, 255). Delta 7-stigmastenol, a recognized plant sterol (256) has also been found in a starfish (257, 258).

*Crustacea*.—Cholesterol has been identified as the sterol of a fresh-water lobster (Malacostraca) (259). A postulated important intermediate in cholesterol biosynthesis, 24-dehydrocholesterol, has been isolated from a barnacle (Entomastraca) and synthesized (260, 261). There appears to be some doubt whether a brine-shrimp should be considered as a primitive entomostracan or malacostracan. It is becoming apparent that evolutionary advancement correlates with increased cholesterol or a change to cholesterol. Cholesterol is the sterol of a brine-shrimp (262) and so from a comparative biochemical viewpoint the brine-shrimp should be considered as primitive on the malacostracan rather than on the entomostracan stem.

*Protochordata*.—Very little is known of the sterols of this class. A tunicate is reported to contain  $\gamma$ -sitosterol, stigmasterol, poriferasterol and 7-dehydrostigmasterol (263). The identifications are based almost entirely on physical properties.

*Coelenterata*.—Two cold-water sea anemones are reported to contain predominately cholesterol (264), while another sea anemone has been shown to have 24-methylencholesterol (214). The physical properties of still another sea anemone are reported to be consistent with those of  $\beta$ -sitosterol (265).

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# CARBOHYDRATE METABOLISM<sup>1,2</sup>

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## INTRODUCTION

The material of this survey has been organized insofar as possible around the framework of various pathways of carbohydrate metabolism, old and new. Coverage by pathways has the advantage that ideas can be organized around reaction mechanisms but has the disadvantage that it tends to emphasize the qualitative rather than quantitative aspects of metabolism. In addition, this orientation coupled with limitations of space has made it necessary to touch on certain important areas of carbohydrate metabolism lightly or not at all. Such areas include the transport of sugars, hormonal controls, methodology as such, and many aspects of oligosaccharide and polysaccharide metabolism.

## PENTOSE PHOSPHATE PATHWAY

The pentose phosphate (pentose-P) pathway may be considered to comprise the reactions leading to the production of ribulose-5-phosphate from glucose-6-phosphate and the subsequent rearrangements of pentose leading to the resynthesis of hexose phosphates. The existence of all or part of this pathway has now been demonstrated in many animal tissues, microorganisms, and plants. The pathway has been considered most often as an alternative route for energy production from glucose-6-P but can be viewed equally well as a mechanism for the production of TPNH, pentose, erythrose-4-P, glycolyl residues, and under some circumstances as a mechanism for the conversion of pentoses to hexoses. When the pentose phosphate pathway is examined as a possible source of a product such as TPNH, it is apparent that the quantitative contribution of this pathway might vary considerably depending on the rate of utilization of TPNH. As DeDuve & Hers (1) have pointed out, the wide variations in the estimates of the metabolism of glucose via the pentose-P pathway may be due partially to this cause. It is interesting to note that in tissues such as liver and mammary gland where TPNH

<sup>1</sup> The survey of literature pertaining to this review was completed November 1, 1957.

<sup>2</sup> The following abbreviations are used in this chapter: ADP for adenosine diphosphate; ATP for adenosine triphosphate; DPN<sup>+</sup> for diphosphopyridine nucleotide, TPN<sup>+</sup> for triphosphopyridine nucleotide; CoA for coenzyme A; UDP for uridine diphosphate; UTP for uridine triphosphate; UDPG for uridine diphosphate glucose; UDPGal for uridine diphosphate galactose; PP for pyrophosphate; P for phosphate; and TPP for thiamine pyrophosphate. Glucose-U-C<sup>14</sup> is used to designate uniformly labeled glucose.

<sup>3</sup> The author is grateful to Drs. H. G. Wood and H. Z. Sable for stimulating discussions and criticisms of the manuscript.

may be required for fatty acid synthesis (2) or hexose synthesis (3, 4), the pentose-P pathway has been found to be of considerable quantitative significance.

*Quantitative evaluation.*—A number of additional attempts have been made to establish the relative contribution of the pentose-P cycle in systems ranging from cells to the intact animal. With one exception, these studies have as their basis the relative isotopic contribution of various carbons of the glucose molecule to various metabolic products. By now the difficulties inherent in the use of isotopic techniques for this purpose are well recognized (5, 6) and the data obtained cannot usually be considered conclusive. Nevertheless, many of these studies are of great interest as a semiquantitative measure of the pentose-P and Embden-Meyerhof pathways.

Dawes & Holms (7) have used the conversion of glucose-1-C<sup>14</sup> and glucose-6-C<sup>14</sup> to CO<sub>2</sub> and pyruvate to evaluate the role of the pentose cycle in *Sarcina lutea*. From the amount of pyruvate obtained from glucose-1-C<sup>14</sup> in the presence of arsenite, they estimate that about 50 per cent of the glucose was metabolized via the pentose-P pathway and the remainder by the Embden-Meyerhof scheme since the Entner-Doudoroff pathway (8) seemed to be ruled out by the position of the C<sup>14</sup> in the pyruvate. Villavicendo & Barron (9) have employed a variety of techniques with lymphatic cells from rabbit appendix and lymphosarcomal and ascites tumor cells. The techniques included the measurement of enzymes, the metabolism of glucose with iodoacetate present to inhibit the Embden-Meyerhof pathway, and studies with variously labeled glucose and lactate. The enzymatic and inhibitor studies suggest that the tumor cells possess an active pentose cycle and that lymphatic cells do not. The studies on the conversion of glucose-1-C<sup>14</sup>, glucose-6-C<sup>14</sup> and glucose-U-C<sup>14</sup> to lactate and CO<sub>2</sub> using the method of Blumenthal *et al.* (10) also indicate that lymphosarcomal cells metabolize glucose via the pentose-P pathway (22 per cent) while lymphatic cells do so only to a minor extent (6 per cent). When the method of Bloom *et al.* (11) using 3, 2, or 1-C<sup>14</sup> lactate was employed with the same cells, the calculated values were 62 per cent for lymphosarcoma and 0 per cent for lymphatic cells; an excellent illustration of the variability of results obtained when different methods are used.

Abraham *et al.* (12) have studied the influence of insulin on the pentose cycle in isolated mammary gland by use of the conversion of glucose-1- and -6-C<sup>14</sup> to CO<sub>2</sub> and fatty acids. Although insulin speeded up the utilization of glucose, the proportion metabolized by the pentose-P pathway remained essentially unchanged. Ashmore *et al.* (13) have compared the contributions of the pentose-P pathway in liver slices from normal and diabetic rats using labeled glucose and fructose. Their results indicate a slight increase in the proportion of glucose metabolized via pentose-P in the liver from diabetic animals although glucose-6-P dehydrogenase was significantly lower in the liver from such animals.

Murphy & Muntz (14) have measured the contribution of the pentose-P pathway with perfused rat liver under conditions which circumvent many

of the assumptions necessary in other studies [cf. Wood (5)]. Rat livers were perfused continuously with fresh blood to which was added successively glucose-6-C<sup>14</sup>, glucose-1-C<sup>14</sup>, and glucose-6-C<sup>14</sup>. Perfusion with each sugar was continued until the radioactivity of CO<sub>2</sub> attained a steady state in each case. By measuring the relative increases or decreases of radioactivity in the CO<sub>2</sub> when the labeled sugars were changed, the proportion of glucose metabolized to CO<sub>2</sub> via pentose-P was estimated at about 55 per cent. Since a steady state was reached and the products of glucose metabolism were not recirculated, the principal assumptions involved are that the contribution of the 6-position of glucose to CO<sub>2</sub> is proportional to the amount of glucose utilized by each pathway and that the pathways have a pool of common intermediates from the triose phosphate stage to CO<sub>2</sub>. As mentioned earlier, the relative importance of the pentose-P pathway may depend upon the metabolic activities of the organ under the conditions of the experiment. It would be extremely interesting to compare the foregoing studies with similar ones in which a substrate for a synthetic reaction, e.g., lactate, was included. It should be noted that the paradoxical situation might exist where glucose oxidation via the pentose cycle might be required for the synthesis of glycogen or glucose from lactate. Early experiments on the formation of glycogen from lactate-C<sup>13</sup> in rats showed that the simultaneous administration of glucose not only greatly increased the yield of glycogen but also markedly stimulated the incorporation of C<sup>13</sup> into glycogen (15, 16). These experiments are consistent with the hypothesis that glucose metabolism is necessary for glycogen synthesis from substances at the lactate-pyruvate level. It has been suggested (3, 4) that the conversion of pyruvate to phosphopyruvate by way of malate requires TPNH. The oxidation of one molecule of glucose via the pentose-P cycle would provide twelve molecules of TPNH, enough to synthesize twelve molecules of phosphopyruvate or six of glucose.

Hers (17) has shown that the C<sup>14</sup>O<sub>2</sub> production from glucose-1-C<sup>14</sup> by rabbit liver slices is increased by the presence of substrates capable of oxidizing TPNH. An interesting case similar to this has been reported by Kinoshita (18) in studies with bovine corneal epithelium. With glucose-1-C<sup>14</sup>, the production of C<sup>14</sup>O<sub>2</sub> was increased eightfold by the addition of a TPNH acceptor, in this case, pyruvate. A similar but lower increase in C<sup>14</sup>O<sub>2</sub> from glucose-2-C<sup>14</sup> was also noted in the presence of pyruvate but with glucose-6-C<sup>14</sup> little C<sup>14</sup>O<sub>2</sub> was produced in either the presence or absence of pyruvate. As a corollary of these experiments, it was shown that only one-half as much glucose-1-C<sup>14</sup> was converted to pyruvate and lactate when pyruvate was added, indicating that the Embden-Meyerhof pathway had been curtailed as the pentose pathway increased. A recent report by Siperstein & Fagan (19) suggests that the synthesis of fatty acids and cholesterol from acetate by rat liver homogenates is markedly stimulated by the addition of glucose-6-P or isocitrate and TPN<sup>+</sup>, presumably by supplying TPNH. The effect is particularly notable in preparations from alloxan-diabetic animals. Although the pentose-P pathway was not demonstrated directly to play a part in the synthesis of fatty

acids and cholesterol, it is implicated circumstantially.

In another series of studies, Muntz & Murphy (20) performed very short time experiments with the injection of variously labeled glucose into the portal vein followed by measurement of radioactivity in the  $\text{CO}_2$ , lactate, and glucose-6-P from the liver. The results confirm the general picture of an active pentose cycle. Black *et al.* (21) have made an interesting attempt to evaluate the pentose pathway in the intact lactating cow by comparing the relative contribution of glucose-1- and -6- $\text{C}^{14}$  to  $\text{CO}_2$  and to glycerol, serine, glutamate, and alanine from milk fat or casein. The  $\text{C}^{14}\text{O}_2$  production was much greater from glucose-1- $\text{C}^{14}$  than from glucose-6- $\text{C}^{14}$ . During the first 24 hours the 6-position of glucose contributed 2.0-2.7 times as much  $\text{C}^{14}$  to glycerol and serine as did the 1-position while the ratio for alanine was lower. It could be calculated that the pentose cycle accounted for 37 per cent to 80 per cent of glucose metabolism depending on the compound chosen as the indicator. The estimates of the quantitative contribution of the pathway serve again to illustrate the difficulties of the determinations but there seems little doubt that the pentose-P cycle has been shown to play a role in an intact animal. It is interesting to note that previous studies with the rat (22) did not indicate such a role. The discrepancy may be due to species or experimental differences, but it is tempting to believe that the difference is due to the large role played by the mammary gland in the metabolism of glucose in the lactating cow. This organ has been shown by several previous studies to have an active pentose-P cycle (23, 24).

*Occurrence of the pentose phosphate pathway.*—Perhaps because of the uncertainties of the isotopic detection of this pathway, an increasing number of studies have relied upon the demonstration of the pathway by enzymatic methods. Despite the complexity of substrate rearrangements, as reflected by tracer experiments, only some six enzymes are unique to the cycle itself: glucose-6-P and 6-phosphogluconic dehydrogenases, transaldolase, transketolase, ribose-P isomerase, and the epimerase which forms xylulose-5-P from ribulose-5-P.

In one of the more interesting extensions of the pentose-P cycle, Cynkin & Gibbs (25) have reported that various members of the genus *Clostridium* can metabolize pentoses. Their data suggest that ribose-5-P can be transformed to glucose-6-P by way of the transketolase-transaldolase sequence. No evidence was obtained for glucose-6-P dehydrogenase, suggesting that in these organisms the pentose-P cycle is only partially complete and serves only to introduce pentoses into hexose metabolic pathways. Various enzymes of the pathway have been demonstrated in saprophytic molds (26) and *Propionibacterium pentosaceum* (27), and DeLey (28) has made an extensive study of the bacterial family, Enterobacteriaceae, and has shown that 22 members of this family contain enzymes of this pathway. Vloedman *et al.* (29) have shown the presence of several of the required enzymes in the ciliate, *Paramecium aurelia*. Also on the basis of enzymatic studies, it can be conjectured that the pentose cycle may play a part in muscle metabolism in the



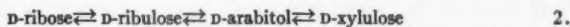
roundworm (30), ascaris, and in the housefly (31), which is in contrast to most studies with mammalian muscle (32).

*Enzymes of pentose phosphate pathway.*—*Aspergillus flavus-oryzae* has been postulated to contain DPN<sup>+</sup>-dependent as well as TPN<sup>+</sup>-dependent glucose-6-P and 6-phosphogluconic dehydrogenases on the basis of different rates of formation of the enzymes during growth (33). No evidence for the participation of transhydrogenase was obtained. It has been reported that *Aspergillus niger* (34) requires Zn<sup>++</sup> in the culture medium for the production of the above two dehydrogenases. A kinase for D-gluconate has been purified from hog kidney (35), the first demonstration of this enzyme in mammalian tissues and the epimerase converting xylulose-5-P to ribulose-5-P has been purified from a mammalian tissue, spleen (36). DeLey & Verhofstede (37) have made the suggestion that 2-ketogluconate-6-P is on a side path of the pentose-P cycle leading from 2-ketogluconate to 6-phosphogluconate. An enzyme which reduced 2-ketogluconate-6-P in the presence of TPNH was purified partially and separated from 6-phosphogluconic dehydrogenase. Ribulose-5-P has been identified as the product of 6-phosphogluconic dehydrogenase of *Escherichia coli* (38) in agreement with similar findings in yeast (39) and liver (40).

Racker & Schroeder (41) have reported the formation of a compound tentatively identified as an octulose-8-P by the action of transaldolase as shown below:

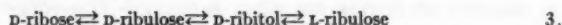


*General interrelationships of hexose and pentose metabolism.*—From the interrelationships of the pentose-P cycle it is clear that under certain circumstances pentoses may be converted to hexoses. Hiatt (42) has studied the formation of liver glycogen in mice using 1-C<sup>14</sup>-labeled D-ribose, D-xylose, and L-arabinose. D-Ribose-1-C<sup>14</sup> contributes about 10 per cent of its C<sup>14</sup> to glycogen, a conversion comparable to that of glucose or galactose while D-xylose was converted only about one-tenth as well and arabinose to a negligible degree. The labeling in the glycogen formed from either ribose-1-C<sup>14</sup> or xylose-1-C<sup>14</sup> was in the C-3 and C-1 positions predominantly, which fits the idea of a transketolase-transaldolase sequence. The relative activities of C-3 and C-1 of the hexose moiety of glycogen were dependent upon the pentose administered and upon the presence or absence of a pentose or hexose pool. Van Sumere & Shu (43) have studied the conversion of pentoses to glucosan in *A. niger*. With D-xylose-1-C<sup>14</sup> the labeling was highest in C-1 with C-3 next in accordance with formation of hexose by the transaldolase-transketolase series. D-Ribose-1-C<sup>14</sup>, unlike xylose gave labeling in C-6 of glucosan hexose as well as C-3 and C-1. The authors suggest that D-ribose is converted to D-xylulose by the following series of reactions before phosphorylation:



The D-xylulose thus formed would be labeled in the 5-position, eventually

giving rise to labeling in the C-6 of glucosan. Another pathway which might be considered is the following:



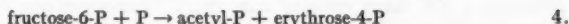
L-Ribulose has been shown recently to be converted to D-xylulose-5-P by reactions discussed in the following section. This type of conversion must be of limited significance in mice since Hiatt (42) found little labeling in the C-6 of glycogen after administration of D-ribose-1-C<sup>14</sup>.

There is continuing interest in the mechanism(s) by which ribose and deoxyribose are synthesized. Marks & Feigelson (44) have compared the labeling of nucleic acid ribose and glycogen in rats fed C<sup>14</sup>O<sub>2</sub> or glucose-2-C<sup>14</sup>. The labeling in the ribose (C-3 > C-2 > C-1) from C<sup>14</sup>O<sub>2</sub> is postulated to be due to a combination of 6-phosphogluconate decarboxylation and rearrangements via the transketolase-transaldolase reactions. It is interesting to note that in the C<sup>14</sup>O<sub>2</sub> experiments, the labeling in glycogen was almost entirely in C-3 and C-4. Bagatell *et al.* (45) have carried out experiments with *E. coli* grown on acetate-1-C<sup>14</sup> in which the RNA ribose and hexose from the glucosan show labeling closely resembling that found by Marks & Feigelson (44). The discrepancy in the labeling of the ribose and polysaccharide hexoses can be explained by assuming that these compounds are drawn from different pools of hexose phosphates, either in terms of time or cellular location. However, it is also possible to explain this discrepancy in terms of the pentose-P reactions if certain assumptions are made. As Horecker & Mehler (46) have pointed out, fructose-6-P labeled in the 3, 4 positions can give rise to pentose with labeling in the C-1, C-2, C-3 in the ratios 33:33:100 by the transketolase-transaldolase sequence. By superimposing the oxidation of glucose-6-P to pentose-P, additional labeling can enter the C-2 of pentose-P, providing a close fit with the observed data. If these reactions are assumed to flow primarily in the direction hexose to pentose, there will be little resynthesis or randomization of C<sup>14</sup> in the fructose-6-P carbons.

Several preliminary reports on the mechanism of formation of deoxyribose have appeared. *Bacillus cereus* can form deoxyribose-5-P from ethanol in the presence of a source of triose phosphate (47), presumably by way of acetaldehyde and the reaction first reported by Racker (48). Homogenates of liver and hepatoma also can synthesize deoxyribose-5-P by the same reaction (49). The origin of the deoxyribose of DNA of rat liver has been studied with C<sup>14</sup>-glycine (50) and these data also conform to the Racker reaction. However, studies with *E. coli* with labeled glucose (51, 52) suggest that the pentose-P pathway is involved in deoxyribose synthesis. Also, in the studies with *E. coli* grown on acetate-1-C<sup>14</sup> mentioned above (45), the deoxyribose labeling corresponds very closely to that of ribose, i.e., C-3 > C-2 > C-1, suggesting that the pentose-P cycle is involved and that deoxyribose may be formed from ribose or that these compounds have a common precursor.

*Reactions related to the pentose phosphate pathway.*—Studies with a mutant of *Acetobacter xylinum* have shown the presence of an enzyme which carries

out a phosphorolytic split of fructose-6-P as shown below (53).



This enzyme also splits xylulose-5-P to acetyl-P and glyceraldehyde-3-P, a reaction also carried out by an enzyme from *Lactobacillus pentosus* (54) although the latter enzyme does not attack fructose-6-P. These phosphorolytic reactions can be added to the relatively small number of mechanisms whereby inorganic phosphate can be esterified.

The conversion of L-arabinose to L-ribulose and to L-ribulose-5-P by pentose adapted organisms was first suggested by Mitushashi & Lampen (55).



A kinase capable of phosphorylating L-ribulose has been purified from *Aerobacter aerogenes* (56) and *Lactobacillus pentosus* (57). The kinase is inactive on L-arabinose, D-xylose, D-ribose, and D- or L-xylulose (57). In addition, a stereoisomerase has been found in the same organisms (57, 58) which converts L-ribulose-5-P to D-xylulose-5-P by inversion at C-4, thereby completing the pathway for the introduction of L-arabinose to the pentose-P pathway. The stereoisomerase has been purified perhaps two hundredfold (58) and has been shown to be free of the C-3 epimerase which interconverts D-xylulose-5-P and D-ribulose-5-P.

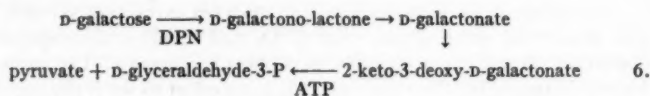
By the use of partially purified and dialyzed extracts of spinach, Mayaudon *et al.* (59) have shown that  $\text{C}^{14}\text{O}_2$  and ribulose diphosphate- $\text{C}^{14}$  are converted almost quantitatively to 3-phosphoglycerate. The same enzyme has been studied by Hurwitz *et al.* (60) in an effort to see if the ene-diol form of ribulose diphosphate is an intermediate. Carboxylation experiments conducted in the presence of  $\text{D}_2\text{O}$  showed considerable incorporation of isotope into 3-phosphoglycerate in accord with the idea that the ene-diol compound is an intermediate but the results as presented must be considered as preliminary.

In spite of the widespread acceptance of ribulose diphosphate carboxylase as the primary enzyme of  $\text{CO}_2$  fixation, some doubts remain. As pointed out by Racker (61), this enzyme has both low activity and low affinity for  $\text{CO}_2$ . Metzner *et al.* (62) have recently presented evidence for the existence of a very labile  $\text{CO}_2$ -fixation product in algal cells which was previously undetected because of the method used in killing the cells. The nature of this product is unknown but the authors estimate that it is formed in amounts considerably larger than the ribulose diphosphate present could account for which suggests the fixation product may not be a derivative of that compound. Warburg *et al.* (63) have suggested that the glutamate- $\gamma$ -aminobutyrate system may play a role in  $\text{CO}_2$  fixation in chlorella. Warburg *et al.* (64) have also compared the rate of formation and disappearance of  $\text{C}^{14}$ -aspartate, glutamate, and alanine from  $\text{C}^{14}\text{O}_2$  in chlorella during weak illumination and have found that all these substances but particularly aspartate rapidly acquire  $\text{C}^{14}$ . The preliminary results on the whole do not seem to support the

hypothesis that glutamate is the first product of  $\text{CO}_2$  fixation. Boichenko & Zakharova (65) have claimed that the  $\text{C}^{14}$  concentration ordinarily used in  $\text{C}^{14}\text{O}_2$  fixation experiments is high enough to inhibit the normal primary fixation processes which they believe to involve the formation of a series of trisaccharides containing saccharic, uronic acids, and ketoses complexed with iron and phosphorus.

**Entner-Doudoroff pathway.**—The inducible fermentation of gluconate and 2-ketoglutarate in *Streptococcus faecalis* has been studied by Sokatch and Gunsalus (66) using gluconate-1- and -2- $\text{C}^{14}$ . Gluconate-1- $\text{C}^{14}$  yielded approximately half its  $\text{C}^{14}$  as  $\text{CO}_2$  and half as lactate-1- $\text{C}^{14}$  while gluconate-2- $\text{C}^{14}$  gave little  $\text{C}^{14}\text{O}_2$  and lactate labeled  $\text{C-2} > \text{C-1} > \text{C-3}$ . The data are best explained by the simultaneous operation of the Entner-Doudoroff (8) and pentose pathways. Glucose metabolism in *Mallomyces pseudomallei* (67) has been suggested on the basis of indirect evidence to involve the Entner-Doudoroff pathway. In *Pseudomonas fluorescens*, 2-keto-gluconate has been shown to enter the Entner-Doudoroff pathway after phosphorylation by a kinase (68) and reduction to 6-phosphogluconate by a reaction analogous to that suggested by DeLey & Verhofstede (37).

DeLey & Doudoroff (69) have reported an interesting variation of the Entner-Doudoroff pathway in galactose fermentation by *Pseudomonas saccharophila* as shown below:



The first reaction is carried out by an inducible enzyme and is similar to the reaction with arabinose by the same organism although the enzymes are probably different. The lactone has been isolated and its hydrolysis shown to be enzymatic. D-Galactonate is converted to the 2-keto-3-deoxy derivative by a reaction analogous to the dehydration of 6-phosphogluconate (8) and arabonate (70) but three different enzymes are involved in the three reactions. The cleavage reaction occurs in several steps in an unknown sequence.

#### GLUCURONATE-XYLULOSE PATHWAY

During the preceding year the final links have been established in a second hexose-pentose cycle which is characterized by the loss of C-6 of the hexose and by the fact that many of the intermediates are non-phosphorylated. This pathway, which appears to be closely linked to the formation of L-ascorbate, connects with the pentose-P cycle through D-xylulose-5-P. This compound appears to rival glucose-6-P as a focal point of carbohydrate metabolism since it has already been shown to be the junction point on the L-arabinose pathway in addition to its important role in the pentose-P pathway.

Some of the interrelationships which will be discussed in the following section are outlined in Figure 1.

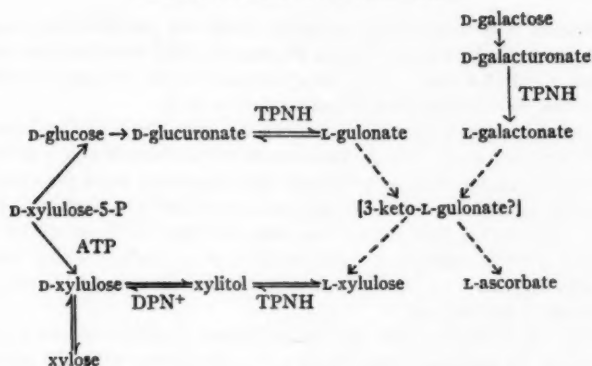


FIG. 1. Possible relationships of D-glucuronate, D-galacturonate, L-ascorbate, and L-xylulose.

**Conversion of glucose to L-gulonate and ascorbate.**—The hypothesis of Isherwood *et al.* (71) that ascorbate is synthesized from glucose via D-glucuronate and L-gulonate has received additional experimental support. This pathway has now been implicated in *A. niger* (72), where the addition of glucuronolactone stimulated ascorbate production three-fold. Thus, the general mechanism has now been suggested to operate in plants, animals, and microorganisms. The conversion of glucose to glucuronate presumably occurs by oxidation of UDP glucose to UDP glucuronate (73) as will be discussed in a later section. The biosynthesis of L-gulonate from D-glucuronolactone-6-C<sup>14</sup> has been studied in rats and guinea pigs (74, 75). Approximately 1 to 3 per cent of the administered lactone was recovered as urinary L-gulonate. Rats treated with either chloretone or barbital were also shown to form gulonate from D-glucose-1-C<sup>14</sup>. The absorption and metabolism of the lactone and salt of glucuronic acid have been compared by Breckenridge & Freeman (76) with the finding that the lactone is both absorbed and metabolized more readily than the salt. Although the lactone is often conceded to be the active intermediate in ascorbate synthesis, Bublitz *et al.* (77) report that in *in vitro* studies the lactone is reduced to L-gulonate by TPNH only after hydrolysis. The explanation for this discrepancy is not obvious at present. Chatterjee *et al.* (78) have reported that the synthesis of ascorbate from glucuronolactone (but not glucuronate) by rat liver homogenates is stimulated by cyanide. Eisenberg (79) has studied the metabolism of D-glucuronate-C<sup>14</sup> *in vivo* in the rat and has found very wide variations in the conversion of this compound to CO<sub>2</sub> (3.5 to 73 per cent). The variations were greatly minimized by nephrectomy suggesting that variable excretion was responsible for the widely differing results in the intact animal.

Various hypnotics such as paraldehyde, barbiturates, and chloretones are known to cause an increase in the urinary excretion of L-ascorbate. The possibility that the increased L-ascorbate excretion resulted from a general

stimulation of a detoxification pathway involving glucuronides has been made less probable by the findings of Burns *et al.* (80) that barbitol which is not metabolized also causes a marked increase in the urinary excretion of ascorbate and of unconjugated D-glucuronate in rats.

The conversion of galactose to ascorbate as also proposed by Isherwood *et al.* (71) has been supported by two enzymatic studies. Mapson & Breslow (81) have purified L-galactose- $\gamma$ -lactone dehydrogenase from peas and have tentatively classified the terminal enzyme in ascorbate production as a flavo-protein. Ashwell (82) has purified an enzyme from rat liver which in the presence of TPNH converts D-galacturonate to a product which has been tentatively suggested to be 3-keto-L-galactonate since it yields L-xylulose after mild acid hydrolysis.

Grollman & Lehninger (83) have made an enzymatic study of the ascorbate pathway in various animal tissues. In accordance with the scheme in Figure 1 they assayed: (a) the enzyme which converts D-glucuronate to L-gulonate (84); (b) the enzyme which converts L-gulonate in the presence of DPN<sup>+</sup> to an intermediate, suggested to be 3-keto-L-gulonate, and (c) an enzyme presumably converting the intermediate to L-ascorbate. The third enzyme has been assayed only in combination with the second since the intermediate is hypothetical. Liver tissue from a number of mammalian species contained all three enzymes while kidney and spleen contained the first two. In chickens, pigeons, and turtles, kidney contained the entire complement of enzymes while liver contained only the first two. In species requiring dietary ascorbate, the first two enzymes were found in liver but the third enzyme was not found in any tissue, which suggests that it is the missing component of the ascorbate synthesizing system. Interestingly, no adrenal tissues from any species tested contained any of the three enzymes. Burns *et al.* (85) also suggest that the missing step of ascorbate synthesis lies beyond L-gulonate.

It is interesting to note that the 3-keto derivatives of L-galactonate and L-gulonate, proposed as intermediates in ascorbate synthesis from L-galactonate (82) and L-gulonate (83), respectively, are the same compound. If this hypothetical intermediate could be confirmed for both pathways it would furnish the key to an interesting relationship between the galactose and glucose pathways to L-ascorbate and L-xylulose as shown in Figure 2.

Although the hypothesis summarized in Figure 2 is an attractive one, it should be emphasized that the key intermediate is entirely hypothetical and quite possibly sufficiently unstable to rearrange spontaneously to L-ascorbate. Mapson & Breslow (81) have not reported any intermediate in the conversion of L-galactonate to L-ascorbate and the results of Grollman & Lehninger (83) on the conversion of L-gulonate to L-ascorbate by two enzymes acting in series can be interpreted in other ways. Since the product of L-gulonate oxidation by DPN<sup>+</sup> was not identified it might have been L-xylulose, and it is conceivable that the second and third enzymes described by Grollman & Lehninger might have a relationship analogous to the oxidation of malate to oxalacetate by malic dehydrogenase and the oxidation of malate to pyruvate and CO<sub>2</sub> by malic enzyme.



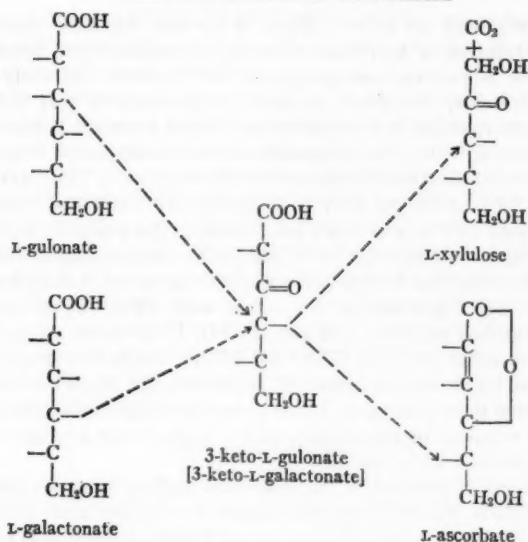


FIG. 2. Possible role of 3-keto-L-gulonate in the synthesis of L-ascorbate and L-xylulose.

Loewus & Jang (86) have reported that cross seedlings (etiolated) like strawberries (87) have a pathway of L-ascorbate formation differing from that discussed above since glucose-1-C<sup>14</sup> gives ascorbate labeled predominantly in the 1-position while the foregoing pathways involve a reversal of numbering of the carbon chain with glucose-1-C<sup>14</sup> labeling the 6-position of ascorbate.

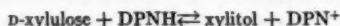
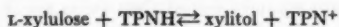
Ascorbate metabolism in guinea pigs has been studied by Salomon (88) by following the rate of ascorbate-1-C<sup>14</sup> disappearance. These studies indicate that the half-life of ascorbate is approximately the same in scorbutic, diphtheria toxin-treated and normal animals. Chan *et al.* (89) have studied the metabolism of variously labeled ascorbate and report the formation of C<sup>14</sup>O<sub>2</sub> from ascorbate-1-C<sup>14</sup> and of C<sup>14</sup>-xylose from ascorbate labeled in other positions. These results could be interpreted as an indication that the final step leading to ascorbate formation is reversible.

**Formation of xylulose.**—It has been shown that the administration of D-glucuronolactone to pentosuric humans increases the yield of L-xylulose in the urine while normal humans and guinea pigs also show small increases in L-xylulose under similar circumstances (90). By analogy with the conversion of D-glucuronolactone and L-galactulolactone to ascorbate, the xylulose should be formed by the loss of the —COOH group. Touster *et al.* (91) have verified this hypothesis by the administration of D-glucuronolactone-1-C<sup>13</sup> to a pentosuric human and the subsequent isolation of L-xylulose-5-C<sup>13</sup>. In



an *in vitro* study with rat kidney, Burns & Kanfer (92) have also demonstrated the formation of L-xylulose from D-glucuronolactone. With the 6-labeled material 30 per cent was converted to  $C^{14}O_2$  while uniformly labeled material yielded only one-sixth as much. Rabinowitz & Sall (93) have studied the same reaction in the supernatant liquid from rat kidney homogenates and have shown by the comparison of glucuronolactone-1- and -6- $C^{14}$  that most of the labeling in  $CO_2$  arises from the 6-position. The reaction was stimulated by TPP, ATP, and UTP as well as by DPN and  $Mg^{++}$ . Although a role of ATP and UTP has not been established in this pathway beyond the glucuronate stage, their stimulation of the further metabolism of that compound might indicate that uridine derivatives are involved. A dehydrogenase which can convert L-gulonate to L-xylulose with  $DPN^+$  as acceptor has been found in guinea pig heart and kidney (94). In the same study it is reported that the substitution of  $TPN^+$  for  $DPN^+$  results in the conversion of L-gulonate to L-glucuronate. Since the D-glucuronate, L-gulonate reaction has been reported to be reversible (77), it seems more logical that the product should be the D-isomer of glucuronate and a more complete report of this work will be awaited with interest.

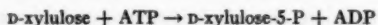
*Interconversion of pentoses.*—The reactions leading from L-xylulose to the pentose cycle in Figure 1 were postulated by Touster *et al.* (95) to account for the glucogenic ability of L-xylulose. The interconversion of the L- and D-xylulose via xylitol has been studied by Hollman & Touster (96) in guinea pig liver mitochondria. Enzymes were obtained by butanol treatment which carried out the following reactions:



7.

The TPNH enzyme is relatively specific while the DPNH enzyme reacts with a number of sugars other than D-xylulose. It is apparent that the interconversion of L- and D-xylulose can be accomplished by the two enzymes working together and that a directional impetus toward the D-form can be furnished by a high ratio of  $TPNH/DPN^+$ . This particular mechanism of metabolic control, i.e., the operation on a mutual substrate by  $DPN^+$  and  $TPN^+$  enzymes has also been reported for sorbitol conversion to glucose ( $TPN^+$ ) or fructose ( $DPN^+$ ) by Hers (97). It is interesting to note that in each case the metabolic flow is believed to be in the direction of DPNH and the concomitant product (D-xylulose, fructose). As has been pointed out (1), the relative concentrations of  $TPNH/TPN^+$  and  $DPNH/DPN^+$  found in cells (98) support this idea.

The missing step in the transformation of L-xylulose to an intermediate of the pentose phosphate cycle, the kinase for D-xylulose has been demonstrated in calf liver by Hickman & Ashwell (98). This enzyme has been previously purified from spinach by Stumpf & Horecker (99).



8.

Xylulose-5-P has also been reported to be formed from xylose by the combination of two induced enzymes, an isomerase and a kinase, in genetic variants of *Salmonella typhosa* (100).

The demonstration of a pathway linking glucuronate or its lactone and D-xylulose-5-P provides a second alternative pentose-intermediate pathway for the oxidation of glucose. Little information is available at the moment as to the quantitative significance of this pathway although at first approximation it would appear to be considerably less important than the pentose-P cycle since the formation of  $\text{CO}_2$  from glucose-6- $\text{C}^{14}$  is smaller in many tissues than the formation of  $\text{CO}_2$  from glucose-1- $\text{C}^{14}$ . Ordinarily,  $\text{C}^{14}\text{O}_2$  arising from the 6-position has been considered to represent the contribution of the Embden-Meyerhof and tricarboxylic acid pathway although it is apparent that this assumption will be in error to the extent that the glucuronate-L-xylulose reactions contribute to  $\text{CO}_2$ . It is perhaps worth noting that the discovery of this second pentose pathway is based for the main part on work with mammalian tissues.

#### EMBDEN-MEYERHOF PATHWAY

Interests in this area have shifted mainly to studies at the enzymatic level or to considerations of the internal and external relationships of the multienzyme glycolytic system.

*Pasteur and Crabtree effects.*—Several efforts have been made to use reconstructed systems composed of glycolytic enzymes and mitochondria to elucidate the mechanism whereby oxidative activity depresses glycolysis (Pasteur effect) and glycolytic activity depresses respiration (Crabtree effect). These effects which obviously may be interrelated constitute a long standing puzzle and although many hypotheses have been offered, none has been established firmly.

Aisenberg *et al.* (101, 102) have demonstrated the Pasteur effect in a system composed of the glycolytic enzymes of brain or tumor cells and liver mitochondria. They suggest that in this particular system, the decrease in glycolysis is not due to a competition between the oxidative and glycolytic components for ADP and Pi but rather to the effect of mitochondrial oxidative processes on phosphofructokinase. Schneider *et al.* (103) have also reported a Pasteur effect in a mitochondrial-glycolytic system but they note that the mitochondrial concentration is an important consideration with stimulation of glycolysis at low concentrations and inhibition at high concentrations. The same authors report that mitochondria will inhibit glycolysis even under anaerobic conditions, which suggests that the inhibition may not be correctly labeled a Pasteur effect. Gatt (104) has also shown that the effect upon respiration and glycolysis of a mitochondrial-glycolytic system can be affected by mitochondrial concentration and by the addition of ATPase. Gatt (104) interprets his results in terms of a competition for ADP and for Pi. The Pasteur and Crabtree effects have been demonstrated in ascites tumor cells by Wu & Racker (105) and the results again are inter-

pretable as a competition for ADP and Pi. The Crabtree effect has also been studied in ascites tumor cells by Yushok & Batt (106) and in retina by Cohen (107). In the former studies (106), the Crabtree effect is shown to be abolished by D-glucosone, an inhibitor of glycolysis, while 2-deoxyglucose is as effective as glucose in initiating the effect. In retina (107), the Crabtree effect is found in developing but not adult retina.

In a simpler reconstructed system von Korff & Twedt (108) have studied the interaction of the glycolytic enzymes lactic dehydrogenase and pyruvic kinase with mitochondria during the oxidation of lactate and phosphopyruvate. These substances were not oxidized by the mitochondria unless the appropriate enzymes which convert them to pyruvate were added externally. When  $\alpha$ -ketoglutarate was oxidized by mitochondria, phosphopyruvate was not used, presumably because of competition for ADP between mitochondria and pyruvic kinase. Hochstein (109) reported that tumor mitochondria are able to glycolyze when supplemented by heated supernatant solution or heated yeast extract.

The various results obtained in the foregoing investigations can be rationalized most easily by postulating that the Pasteur and Crabtree effects can arise from several different causes, at least in model systems. It seems likely that more quantitative information on the factors limiting the rates of the particular glycolytic and oxidative systems employed will be required before definitive answers will be available.

*General studies.*—An interesting relationship between the metabolism of ascites tumor cells and the host animal has been demonstrated by Hiatt (110). Glucose- $C^{14}$  was given intraperitoneally to normal and ascites tumor bearing mice and the distribution of the  $C^{14}$  in liver glycogen determined. The results indicate that a cleavage of the sugar had occurred in the ascitic animal while the intact carbon chains of glucose were incorporated into glycogen in the normal animal. In contrast, when glucose- $C^{14}$  was given to ascitic animals by an extraperitoneal route, the liver glycogen showed a noncleavage pattern, presumably because the strong glycolytic activity of the tumor cells had been bypassed. The carbohydrate metabolism of chick embryo heart and liver explants has been studied during the transition from *in vivo* to *in vitro* conditions (111, 112) and in growing and regressing Flexner-Jobling carcinoma (113).

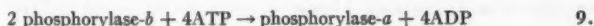
Shaw & Stadie (114) have made the very provocative proposal that rat diaphragms contain two coexisting glycolytic pathways, one insulin responsive and leading from glucose to glycogen, and a second non-insulin responsive pathway leading from glucose to lactic acid. Glycogen formation by rat hemidiaphragms was stimulated by insulin while the production of lactate was not affected by the hormone. Glucose- $C^{14}$  was found to label intracellular glucose-1-P and glucose-6-P but not fructose-1,6-di-P. The incorporation of  $C^{14}$  into the first two esters was accelerated by insulin. Experiments with externally added pools showed that glucose-6-P and fructose-1,6-di-P acquired  $C^{14}$  but that glucose-1-P did not. The postulate is made that the insulin-

responsive pathway leading to glycogen is in effect an intracellular process, while the insulin-insensitive pathway to lactate is in effect a surface process. The possibility that a tissue or cell may contain the same compounds or metabolic pathways in geographically separated locales has been considered by many workers, but the above work is one of the most interesting presentations of experimental evidence from any single tissue purporting to demonstrate such a situation. Because of the far-reaching implications, the interpretations of the above experimental results in terms of coexisting pathways in the same tissue will receive close scrutiny. The possibility that the "external" pathway is due to leached-out enzymes is discounted by the authors on the basis of direct enzymatic tests of the suspending medium which failed to show the presence of most of the glycolytic enzymes, although there is some possibility that the disparity in the timing of the metabolic and enzymatic experiments may furnish a basis for discussion. A second possibility that must be considered is that the diaphragm preparation contained two types of cells, one of which showed the properties of the "internal" pathway and a second type which had the metabolic characteristics of the "external" pathway. Two such types of cells might exist in diaphragm *per se* or be formed by the preparative procedures. It is interesting to note that Kipnis & Cori (115) found that "cut" and "intact" diaphragms showed considerably different permeability properties. For example, the thiosulfate space in "cut" diaphragm was approximately twice that of "intact" diaphragms. Although it may not be accurate to equate the hemidiaphragms used by Shaw & Stadie (114) with the "cut" diaphragm, it would be very valuable to have data from experiments with the intact diaphragm.

Ashmore *et al.* (13) have made study of the relative rates of various reactions which glucose-6-P may undergo in liver slices. These reactions include conversion to glycogen, oxidation by the pentose phosphate pathway, glycolysis, and dephosphorylation to glucose. By the use of labeled glucose and fructose the contribution of each of these pathways is estimated in media of different ionic constitution and in liver from normal and diabetic animals.

*Glycolytic enzymes.*—Many studies of the various glycolytic enzymes have been reported during the last year, ranging from demonstration of the presence of glycolytic enzymes in tissues to studies on the mechanism of action of highly purified enzymes. For the most part, these studies must be considered very briefly.

Cori & Illingworth (116) have reported that muscle phosphorylase-*a* and -*b* contain 4 and 2 moles of pyridoxal P per mole, respectively. The pyridoxal P, whose function is unknown, can be dissociated by acid ammonium sulfate and reactivation can be effected by pyridoxal P but not by pyridoxal or pyridoxamine P. Krebs *et al.* (117) have studied the stoichiometry of the conversion of phosphorylase-*b* and -*a* by the use of ATP<sup>32</sup> and report the following relationship:



The binding of AMP by phosphorylase-*a* and -*b* has been reported to be 4 and 2 moles of AMP per mole, respectively (118). AMP reactivates phosphorylase-*a* after heat inactivation but has no effect on urea inactivation (119).

Studies on the effect of epinephrine and glucagon on the reactivation of liver phosphorylase have been continued by Rall *et al.* (120). In the presence of glucagon or epinephrine, ATP, and a particulate fraction from liver, a heat stable factor was formed which stimulated the reactivation of phosphorylase by ATP and a supernatant fraction from liver. Cook *et al.* (121) have reported the formation of a cyclic dianhydroadenylic acid by treatment of ATP by  $\text{Ba}(\text{OH})_2$ . Sutherland & Rall (122) report that this compound appears to be identical in chemical and biological properties with their heat stable substance. Cahill *et al.* (123) have studied the effects of  $\text{K}^+$  and  $\text{Na}^+$  on glycogen breakdown by liver slices. Through direct measurement of phosphorylase, it was shown that slices in  $\text{Na}^+$  contained considerably more of the active form of this enzyme than slices incubated in  $\text{K}^+$ , thereby leading to more rapid breakdown of glycogen in  $\text{Na}^+$ . The authors suggest that  $\text{Na}^+$  is acting by inhibiting the phosphorylase inactivating enzyme, thereby preserving a larger proportion of active phosphorylase.

The presence of nonspecific hexokinases resembling those of the yeast and muscle has been reported in locust muscle (124), ascites tumor (125), brains from several species (126) and *Neurospora crassa* (127). The tumor and brain hexokinases are at least partially sedimentable. The formation of a hexokinase in *Neurospora* has been found to be partially dependent upon the presence of  $\text{Zn}^{++}$  with a demonstrable activation of the enzyme from Zn-deficient cultures by  $\text{Zn}^{++}$  (127). The sensitivity of pancreatic hexokinase to alloxan has been investigated by Villar-Palasi *et al.* (128). The partially purified enzyme is inactivated by alloxan with glucose exerting a protective effect, but because of the high concentrations of substances required they conclude that this mechanism is probably not involved *in vivo*.

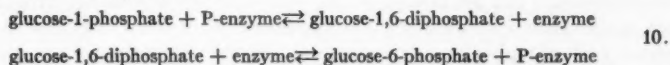
Two groups of investigators have utilized measurements of the  $K_{eq}$  of the hexokinase reaction to recalculate the free energy of hydrolysis of ATP. Robbins & Boyer (129) determined the  $K_{eq}$  of hexokinase by utilizing the incorporation of  $\text{C}^{14}$ -glucose-6-phosphate into glucose while Vladimirov *et al.* (130) used glucose-6- $\text{P}^{32}$  in the same way. The  $-\Delta F^\circ$  for hexokinase was calculated as  $-4.5$  to  $-4.7$  kcal. at pH 7.0 (129) and  $-2.6$  to  $-4.4$  kcal. (130), respectively, and when combined with the  $-\Delta F^\circ$  for the hydrolysis of glucose-6-phosphate to glucose and  $\text{P}_i$ , the  $-\Delta F^\circ$  for the hydrolysis of ATP ranges from  $-7.6$  to  $7.8$  kcals. for the American workers and  $-5.05$  to  $-6.85$  kcal. for the Russian workers.

Liver fructokinase has been highly purified (131) and the  $K_m$  values for ATP, Mg-ATP, fructose, and  $\text{K}^+$  determined. The enzyme is severely inhibited by trace amounts of ADP and measurements were carried out with creatine-P and creatine kinase present to rephosphorylate ADP. It has been reported that the effect of adenosine and its derivatives in protecting

erythrocytes in preserved blood is due to a stabilization effect of these substances on phosphofructokinase (132). Wieland & Suyter (133) have crystallized and described some of the properties of liver glycerokinase. A kinase for D-glyceric acid has been partially purified from liver by two different groups of workers (134, 135).

Harary (136) has made the interesting finding that acetyl phosphatase, whose function in muscle has remained a puzzle, hydrolyzes many acyl phosphate esters including 1,3-diphosphoglyceric acid. Harary points out that in combination with 3-phosphoglyceric kinase, acyl phosphatase can act as an ATPase. Goodlad & Mills (137) present evidence that the hydrolysis of glucose-1-P proceeds via phosphoglucomutase and glucose-6-phosphatase. Langdon & Weakley (138) have solubilized glucose-6-phosphatase from kidney and liver microsomes by digitonin treatment. The purified enzyme is inactive on other hexose phosphates and  $\beta$ -glycerophosphate.

The phosphoglucomutase reaction as formulated below has been studied by Sidbury & Najjar (139):

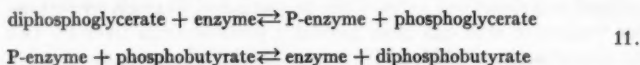


The  $K_{eq}$  for the first reaction has been calculated as 4.56, and for the second 3.76, and the  $\Delta F^\circ$  for the hydrolysis of the P-enzyme bond calculated to be  $-3.9$  kcal. which suggests that phosphoserine may be involved. The possibility that the phosphate moiety is not attached to serine in the native enzyme but rather migrates to serine from an adjacent N atom during acid hydrolysis has not been eliminated completely. However, this possibility is not supported by the experiments of Anderson & Jollès (140) who found no evidence of an N—P binding by the use of 1-fluoro-2,4-dinitrobenzene although they were able to isolate O-phosphoserine and serine peptides. Kennedy & Koshland (141) also present results on  $P^{32}$ -labeled phosphomutase which support the hypothesis that the  $P^{32}$  bond is with serine. Koshland & Erwin (142) have made the exciting finding that phosphoglucomutase marked with  $P^{32}$  yields a hexapeptide containing P-serine which is identical with a hexapeptide isolated from chymotrypsin (143). The hypothesis is advanced that enzymes which carry out similar types of reaction may have a common sequence of amino acids even though the substrate specificities may vary widely. This hypothesis rests upon the assumption that the hexapeptides isolated represent a functional portion of the two enzymes but the implications for enzyme chemistry are large. Phosphoglucomutase has been obtained in highly purified form from potatoes and its properties found to correspond closely to the muscle enzyme (144).

Indirect evidence has been obtained by Pizer & Ballou that a phosphoenzyme is also involved in the action of phosphoglyceric mutase (145). The analogues of 3- and 2-phosphoglycerate, the monophosphate esters of 2,3-dihydroxybutyrate, serve as substrates for phosphoglyceric mutase and 2,3-diphosphobutyrate can replace 2,3-diphosphoglycerate as an activator for the



enzyme. 2,3-Diphosphobutyrate can be synthesized from 2,3-diphosphoglycerate and the mechanism is postulated to be as follows:



However, Rodwell *et al.* (146) have reported that crystalline yeast phosphoglyceric mutase did not form 2,3-phosphoglycerate from 2- or 3-phosphoglycerate as might be expected if the enzyme crystallized as a phosphoprotein. Also incubation of 2,3-diphosphoglycerate with the mutase led to 2- or 3-phosphoglycerate and Pi. The estimation and distribution of 2,3-diphosphoglycerate and its formation from 3-phosphoglycerate and ATP by extracts from various tissues has been studied (147).

Topper (148) has studied the incorporation of deuterium into glucose-6-phosphate during its formation from fructose-6-phosphate by phosphoglucisomerase. The results indicate that 1 mole of deuterium is incorporated on C-2 and are interpreted in terms of a mechanism involving a proton rather than a hydride ion. In experiments with deuterio-glucose-6-phosphate and phosphoglucisomerase and phosphomannoisomerase, the two isomerases appear to be stereospecific for different H atoms on the C-1 of fructose-6-P. Wick *et al.* (149) report that the primary metabolic block produced by 2-deoxyglucose may be due to inhibition of phosphoglucisomerase by 2-deoxyglucose-6-P formed by the action of hexokinase.

Drechsler (150) has degraded aldolase with carboxypeptidase and obtained a product which has only 7 per cent of its original activity although it is indistinguishable from the native enzyme during ultracentrifugation and appears to have lost only tyrosine and threonine in significant amounts. It has been found possible to demonstrate the presence in serum of aldolases of probable liver and muscle origin (151). Titration of the sulfhydryl groups of aldolase with *p*-chloromercuribenzoate has shown 28 —SH groups per mole of enzyme of which up to 10 can be lost without decrease in activity (152).

Extensive studies on the equilibrium and kinetics of enolase have been presented by Wold & Ballou (153, 154). The apparent  $K_{eq}$  is 6.3 (2-phosphoglycerate  $\rightleftharpoons$  phosphopyruvate) and is independent of pH and metal ions according to the mathematical treatment developed by Wold & Ballou (153). Space does not permit an adequate discussion of the many interesting points developed in these studies concerning specificity and mechanism of action. Malmstrom (155) has reported that treatment of crystalline yeast enolase by zone electrophoresis and ion exchange chromatography yields several active forms of the enzyme. The significance of this observation is not yet clear.

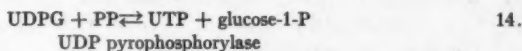
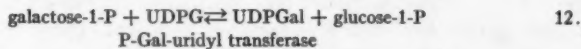
#### METABOLISM OF OTHER COMPOUNDS DERIVED FROM GLUCOSE

This section deals with a number of hexoses and their derivatives which can be formed from glucose and which were not discussed in the preceding



sections. These include galactose and lactose, some of the deoxy sugars, uridine derivatives of hexoses, cellulose, and inositol.

*Galactose-lactose.*—It has now been shown that a defect in the metabolism of galactose-1-P exists in the liver of individuals suffering from congenital galactosemia (156). Previously, it had been shown (157) that lysed erythrocytes of such individuals failed to carry out the P-Gal-uridyl transferase reaction, the first reaction in the following series, which is believed to represent the metabolic path of galactose-1-P (158):

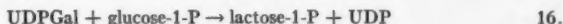
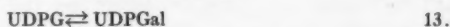
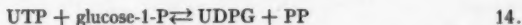


In the liver studies (156) it was necessary to show the defect in a more indirect fashion since the products of the reaction are further metabolized. The incorporation of 1-C<sup>14</sup>-galactose-1-P into the uridine fraction was found to be very low in galactosemic adults and negligible in galactosemic infants. The small incorporation of galactose-1-P into uridine compounds in the galactosemic adult has been further investigated (159) and evidence obtained for an accessory pathway of galactose utilization. Studies with rat liver (160) show the presence of UDPGal pyrophosphorylase.



The amount of this enzyme is very small in fetal liver in rats but increases markedly with age. This observation supplies an attractive hypothesis for the increase in galactose tolerance in galactosemic adults as compared with galactosemic infants. The same enzyme has also been demonstrated in plants (161). Interesting preliminary reports (162, 163) indicate that galactose-1-P inhibits phosphoglucomutase, suggesting a possible explanation for the known toxic effects of galactose in galactosemic individuals or even in normal individuals when administered in excess. Kurahashi (164) has investigated the enzymatic defects in mutants of *E. coli* which cannot adapt to galactose. The pathway in this organism is apparently similar to that proposed for mammalian tissues (158). Of seven different mutants, three showed an inability to form galactokinase and four failed to form P-Gal-uridyl transferase (Reaction 12). The relationship of the different mutants in each group is not well understood.

Gander *et al.* (165) have reported more fully on the role of lactose-1-P in lactose synthesis and propose the following mechanism:



Mammary gland extracts were able to convert up to 70 per cent of UDPG and  $C^{14}$ -glucose-1-P to a sugar phosphate which is hydrolyzed under mild conditions to lactose. The sugar is not reducing until hydrolyzed, suggesting that the phosphate is on the 1-position. Glucose-1-P doubly labeled with  $C^{14}$  and  $P^{32}$  was incorporated into the sugar phosphate without appreciable dilution of either label. Under the above conditions, the galactose moiety does not incorporate counts from labeled glucose-1-P suggesting that the above preparation lacks the first enzyme in the foregoing scheme although this enzyme has been demonstrated previously in mammary gland (23).

The synthesis of lactose in lactating animals has been studied by Wood and his co-workers. After intravenous injection of acetate-1- $C^{14}$ ,  $NaHC^{14}O_3$ , or glucose-1- or -2- $C^{14}$ , the labeling in the galactose and glucose moieties of lactose was approximately equal (166) but when acetate-1- $C^{14}$  or propionate-1- $C^{14}$  was administered to the isolated udder by perfusion, 90 per cent of the incorporated label was found in galactose with the C-4 containing three times as much  $C^{14}$  as the C-3 (167). In a unique confirmation of the above findings, Wood *et al.* (168) injected acetate-1- $C^{14}$  into the arterial supply of one side of the mammary gland and showed that milk lactose from the injected side resembled that formed in perfusion experiments apparently reflecting mammary gland metabolism while milk lactose from the uninjected side had approximately equal activities in galactose and glucose and apparently reflected the combined metabolism of the liver and mammary gland. The foregoing results pose two questions which cannot be answered satisfactorily at the present time. If the mechanism proposed by Gander *et al.* (165) is operating, the galactose and glucose moieties should be equally labeled since both arise from the same source, glucose-1-P. In addition, the peculiar labeling of galactose with the bulk of the  $C^{14}$  in the C-4 must be explained. The possible explanations include the formation of UDPGal from a different pool of glucose-1-P than that which reacts with UDPGal; the reaction of labeled UDPGal with relatively unlabeled glucose rather than with glucose-1-P (167); and the possibility that labeled galactose is formed from acetate- $C^{14}$  by a different pathway than the UDPG route with the conversion of galactose to UDPGal by UDPGal pyrophosphorylase (160) (Reaction 15). This enzyme has not been reported in mammary tissue. The experiments of Pazur & Tipton (169) suggest that the galactose moiety of lactose may not be derived entirely from glucose when galactose is present since galactose-1- $C^{14}$  gave higher labeling in the galactose moiety of lactose while glucose-1- $C^{14}$  gave higher labeling in the glucose moiety. Since P-Gal-uridyl transferase has been shown to be absent from mammary tissue (23), this enzyme cannot be involved in the formation of UDPGal. The explanation for the peculiar labeling in galactose has been tentatively suggested to be due to slow triose isomerase activity (168) with dilution of the C-3 and the upper half of the molecule from a source such as glycerol but further experimentation has not supported this hypothesis (170). Another possibility is that the mammary gland cannot convert fructose-1,6-di-P to fructose-6-P by de-

phosphorylation although by the action of transketolase and transaldolase, labeled triose P arising from acetate- $C^{14}$  could enter the lower half of the hexose monophosphates. Venkataraman & Reithel (171) have studied the synthesis of lactose by mammary gland slices and have shown that lactose synthesis does not take place anaerobically although glucose utilization is virtually unchanged. Preincubation of the slices, even in the absence of glucose, increased lactose synthesis.

**Rhamnose-fucose.**—The 6-deoxyhexoses, rhamnose and fucose, are found in many natural materials including blood group substances and bacterial polysaccharides but their metabolism has been poorly understood. During the period covered by this report several studies which bear on this subject have appeared. Wilson and Ajl have shown that the first step in the adaptive utilization of L-rhamnose by *E. coli* is an isomerization to L-rhamnulose (172) followed by a kinase action with ATP to form L-rhamnulose-P (173). The phosphate is suggested to be on the C-1 mainly on the basis of ease of hydrolysis. The further metabolism of rhamnulose-phosphate is unknown but the ester may be converted to dihydroxyacetone-P and lactaldehyde.

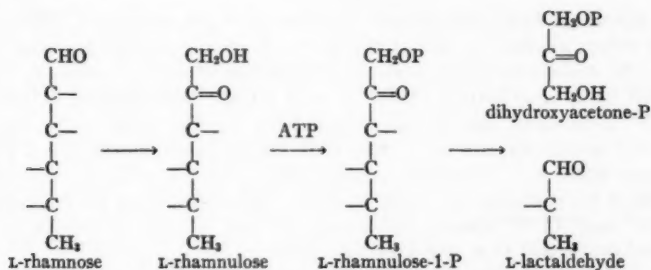


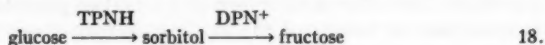
FIG. 3. Possible metabolic pathway for L-rhamnose.

Englesberg (174) has found a compound tentatively identified as lactaldehyde as a product of rhamnose fermentation of a mutant of *Pasteurella pestis*. The biosynthesis of rhamnose and a rhamnolipid by *Pseudomonas aeruginosa* has been studied by Hauser & Karnovsky (175) using glycerol- $C^{14}$ . Glycerol- $\alpha$ - $C^{14}$  gave rhamnose labeled in positions 1, 3, 4 and 6 while glycerol- $\beta$ - $C^{14}$  gave labeling in positions 2 and 5. These results are consistent with the condensation of dihydroxyacetone-P and lactaldehyde to form rhamnose, the reverse of the reaction shown above but the mechanism will not be confirmed until this aldolase-like reaction and its true substrates have been demonstrated.

Two preliminary reports on the synthesis of L-fucose from D-glucose by aerobacter have appeared (176, 177). L-Fucose and D-glucose isolated from the polysaccharides of organisms grown on glucose-1- or -6- $C^{14}$  showed essentially the same labeling with major labeling corresponding to the initial

position of  $C^{14}$  with some randomization into the other half of the hexose (176). In similar studies with glucose-6- $C^{14}$ , 95 per cent of the L-fucose labeling was found in C-6 (177). It is difficult to postulate a mechanism for L-fucose formation which does not involve the condensation of two three carbon fragments but the lack of randomization of the 1 and 6 carbons during the conversion of glucose to fucose would suggest that the triose fragments from the two halves of glucose are not identical and do not become equilibrated to any large extent via triose isomerase or that a nonsplitting mechanism operates in the conversion.

*Sorbitol.*—The metabolism of sorbitol-U- $C^{14}$  and glucose-U- $C^{14}$  in normal and diabetic humans has been studied by Adcock & Gray (178). On the basis of time curves of the specific activities of glucose and  $CO_2$  after sorbitol and glucose feeding, it is suggested that glucose is not an obligatory intermediate in sorbitol metabolism, but rather that sorbitol is converted to fructose as previously suggested (97) for seminal vesicles.



Silkworm eggs have been shown to convert glycogen to sorbitol (179) which may reflect another portion of the proposed glucose-sorbitol-fructose pathway. In *Acetobacter suboxydans* the metabolism of sorbitol seems to be directed by a combination of  $DPN^+$  and  $TPN^+$  dehydrogenases (180). A  $DPN^+$  dependent dehydrogenase has been purified from sonic extracts which converts sorbitol to fructose while there is also evidence for a  $TPN^+$  enzyme which has sorbose as a product (181). This is another interesting example of the oxidation of a sugar alcohol at different ends of the molecule by  $DPN^+$  and  $TPN^+$  specific dehydrogenase and is very analogous to the xylitol conversion to D- and L-xylulose (96).

*Uridine and amino derivatives of sugars.*—Uridine derivatives of hexoses have been shown to participate widely in the interconversions of hexoses and the synthesis of disaccharides and evidence now suggests that they are similarly involved in the synthesis of certain polysaccharides and mucopolysaccharides. The interest in the mechanism of synthesis of mucopolysaccharides has led to many studies on the metabolism of the hexuronic acids and amino sugars which serve as building blocks in these syntheses.

Some of the possible interrelationships involved in the synthesis of hexosamines which will be discussed in this section are summarized on page 267. The synthesis of D-glucosamine-6-P from hexose-6-P and glutamine has been described recently in a rat liver system by Pogell & Gryder (182). A similar reaction was described for neurospora by Leloir & Cardini (183). The substrate of the rat liver system appears to be glucose-6-P rather than fructose-6-P on the basis of changes in relative activity of the two substrates during purification of the enzyme although the results cannot be considered entirely conclusive. This finding is in apparent contradiction to earlier studies with the neurospora enzyme where fructose-6-P appeared to be a more effec-

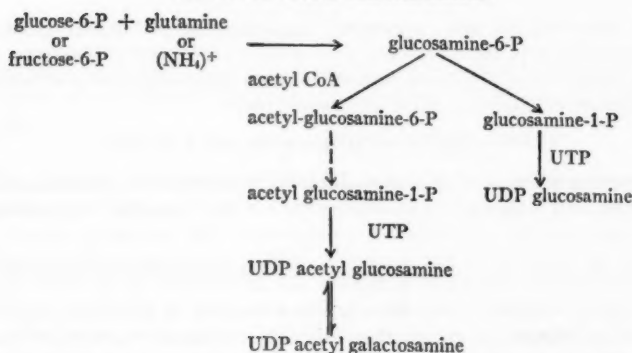


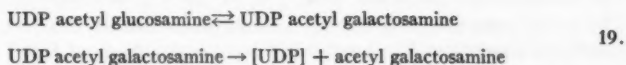
FIG. 4. Some possible interrelationships in hexosamine synthesis.

tive substrate (184). Although glucosamine-6-P can be synthesized by other pathways such as the direct phosphorylation of glucosamine or the reaction of fructose-6-P with  $\text{NH}_4^+$  (185), the glutamine pathway may be an important one in the synthesis of mucopolysaccharides since Roden (186, 187) has shown that glutamine greatly stimulates the incorporation of glucose- $\text{C}^{14}$  into the hexosamine portion of chondroitin sulfate in *in vitro* experiments with cartilage slices. The degradation of glucosamine-6-P to fructose-6-P and  $\text{NH}_4^+$  has been shown in *Pseudomonas* (188), *E. coli* (189) and *Aerobacter aerogenes* (190) with 2-imino-fructose-6-P postulated as an intermediate.

Davidson *et al.* (191) have demonstrated the presence of an enzyme which can acetylate glucosamine-6-P in molds, bacteria, and rabbit tissues. The enzyme from *Neurospora* has been purified 230-fold and requires acetyl CoA. Only rabbit liver of the various sources tested acetylates glucosamine and the rate is only one-third as large as with the phosphate ester suggesting that the phosphorylated form is the usual substrate for acetylation. Roseman (192) has also described an enzyme from *E. coli* which deacetylates N-acetyl glucosamine.

The further metabolism of N-acetyl glucosamine probably involves its uridine derivatives since UDP acetyl glucosamine has been found in such sources as pneumococci (193) and plants (194). Maley & Lardy (195) have shown the formation of UDP glucosamine from UTP and glucosamine-1-P in extracts of rat liver nuclei and of UDP acetyl glucosamine from UTP and acetyl glucosamine-1-P in the supernatant fraction of liver homogenates.

Cardini & Leloir (196) have found that preparations from guinea pig and rat liver can carry out the following reactions:



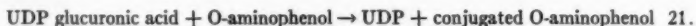
Preparations from *Saccharomyces fragilis* which contain UDPGal-4-epimerase

do not carry out the above reactions, suggesting that a different epimerase may be involved in the transformation of the amino sugar derivatives.

Strominger *et al.* (73) have purified the enzyme from calf liver catalyzing the oxidation of UDPG to UDP glucuronic acid:



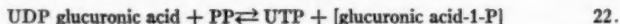
The reaction is irreversible and no intermediates could be detected. UDP glucuronic acid is apparently an intermediate in the formation of glucuronide formation.



Strominger & Mapson (197) have purified the first of these two enzymes from pea seedlings and report that this enzyme closely resembles the one from calf liver. Crude preparations from pea seedlings oxidize UDPGal but the product is UDP glucuronate and the mechanism apparently involves UDPGal-4-epimerase. Since no UDP galacturonic acid was formed, this substance might be formed by a separate epimerase or by a separate UDPGal dehydrogenase analogous to the UDPG reaction. Smith *et al.* (198) have found UDP galacturonic acid in Type I pneumococci and since no UDP glucuronic acid has been found in this organism, they suggest that the mechanism of formation of UDP galacturonic acid involves the dehydrogenase rather than the epimerase mechanism.

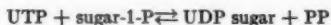
Castellani *et al.* (199) have made the interesting observation that homogenates of epiphyseal cartilage contain the enzymes for the formation of UDPG from UTP and glucose-1-P and for the oxidation of UDPG to UDP glucuronic acid, thereby linking these reactions with mucopolysaccharide synthesis as well as with conjugation reactions. Axelrod *et al.* (200) have shown that the UDP glucuronate can form glucuronic acid conjugates with an N-group (aniline) as well as the earlier described ester and ether conjugates.

Smith *et al.* (201) have compared the uridine derivatives of Type II (non-capsulated) and Type III (capsulated) pneumococci. Both strains were found to contain UDP derivatives of glucuronic acid, acetyl glucosamine, and glucose and in approximately similar quantities. The inference is drawn that the failure of capsule formation is due to steps subsequent to the formation of the apparent building blocks. Solms *et al.* (194) have found UDP glucuronic acid in mung bean seedlings. They also found an enzyme in this source which carries out the apparent reaction:



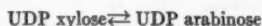
The reaction was measured from left to right with identification of the UTP but glucuronic-1-P is only by analogy. If confirmed, this reaction would constitute a new possible mechanism for the synthesis of UDP glucuronic acid. Neufeld *et al.* (161) have described the following general reaction for mung bean extracts:





23.

where the sugar may be  $\alpha$ -D-glucose,  $\alpha$ -D-galactose,  $\beta$ -L-arabinose,  $\alpha$ -L-arabinose, or  $\alpha$ -D-xylose. The same extracts also carried out an epimerase reaction



24.

although it is not clear whether UDPGal-4-epimerase or a different enzyme is involved.

*Cellulose synthesis.*—*In vitro* studies of cellulose biosynthesis have made much progress recently although space permits only mention of a few of the studies which seem relevant to the foregoing discussion. Glaser (202) recently found that sonic preparations of *Acetobacter xylinum* can incorporate the  $\text{C}^{14}$  of UDPG- $\text{C}^{14}$  into "cellulose" when incubated with celloextrins. In these preparations  $\text{C}^{14}$ -glucose-1-P and glucose- $\text{C}^{14}$  were inactive. However, Greathouse (203) has shown that glucose-1- $\text{C}^{14}$  enters cellulose only when ATP is present and that the  $\text{C}^{14}$  is almost entirely localized in the C-1 of the glucose moiety of cellulose. Colvin (204) also showed that the utilization of glucose by the same organism was dependent on ATP. Schramm *et al.* (205) have discussed the role of the pentose-P pathway in cellulose formation in this organism.

The metabolism of myo-inositol has been the subject of an interesting series of papers by Charalampous and co-workers. The isolation of inositol from yeast (206) and the development of a method of degradation involving iodosaccharic acids (207) were followed by studies on the biosynthesis of myo-inositol from glucose-1,2- or -6- $\text{C}^{14}$  in yeast (208). The pattern of labeling in inositol from these sources is very complex but suggests that cyclization of glucose is not the mechanism but rather that inositol might be formed by a condensation of a tetrose and a 2-carbon unit. The conversion of myo-inositol to D,L-glucuronic acid by supernatant fractions of kidney homogenates has also been described by Charalampous & Lyras (209).

#### TRICARBOXYLIC ACID CYCLE

Despite much interest in alternate pathways, the tricarboxylic acid cycle has been established as a major oxidative vehicle in animal tissue and in many species of microorganisms and plants.

*Occurrence.*—Kleiber and his co-workers have studied the conversion of  $\text{C}^{14}$ -acetate to the glycerol of milk fat (210) and to the amino acids of milk casein in the intact dairy cow (211). With both products, the distribution of  $\text{C}^{14}$  fits well with that predicted for the oxidation of acetate via the tricarboxylic acid cycle. Sonic extracts of HeLa cells grown in tissue culture showed the presence of many enzymes of the tricarboxylic acid cycle (212). Experiments with  $\text{C}^{14}$ -acetate suggest that bone contains a small metabolically active pool of organic acids, and a larger pool coprecipitated with bone salt and metabolically inactive (213). The formation of citrate in the presence of fluoroacetate has been shown for slices of transplantable tumors as



well as slices of many tissues (214). These results demonstrate that under specific conditions all of these tissues including tumor show some of the reactions of the tricarboxylic acid cycle, although they furnish no evidence as to the quantitative role of the cycle under *in vivo* conditions.

The fungus, *Zygorrhysichus moelleri*, when grown on media containing  $C^{14}O_2$  or acetate- $C^{14}$ , shows labeling in glutamate and aspartate in conformity with the tricarboxylic acid cycle (215). In the same work, it is estimated that 60 per cent of the oxalacetate is recycled and 40 per cent formed from  $CO_2$ . Studies on the metabolism of glutamate-3,4- or -1- $C^{14}$  in crown gall indicated that  $\alpha$ -ketoglutarate is formed from glutamate and metabolized to form both tricarboxylic and dicarboxylic acids (216). Since glutamate-1- $C^{14}$  labels citrate, the latter may be formed by reversal of the isocitrate dehydrogenase reaction.

DeMoss & Swim (217) have provided convincing evidence that the tricarboxylic acid cycle plays a major role in the oxidation of acetate in baker's yeast. The intracellular pools of the various intermediates of the cycle were shown to be in near equilibrium with the acetate-2- $C^{14}$  and the distribution of labeling in citrate and succinate was in full accord with the patterns of the cycle. Comparison of labeling in the citrate carboxyls and  $CO_2$  also indicated that it was unlikely that any more direct oxidative pathway for acetate played a quantitatively important role. Previous doubts about the operation of the cycle in intact yeast (218) seem to be answered in the main by this work. Evidence for the tricarboxylic acid cycle has been obtained for *Shigella flexneri* (219) and *Serratia marcescens* (220).

Tyler (221) has proposed that the inhibition of succinate oxidation by oxalacetate may constitute a regulatory mechanism for the tricarboxylic acid cycle. The inhibition was relieved by  $Mg^{++}$  and ATP, presumably by the complexing of these substances with oxalacetate. Oxalacetate has also been shown to inhibit the oxidation of succinate and pyruvate in cytoplasmic particles of avocado (222).

*Enzymes.*—Condensing enzyme has been purified from *Mycobacterium tuberculosis* with properties which resemble those of the pure pig heart enzyme (223). Isocitric dehydrogenase from pig heart has been highly purified (224) and its properties studied (225). Over a wide range of purification, the ratio of isocitric dehydrogenase and oxalosuccinate decarboxylase activities remains constant as does the relative activity of oxalosuccinate as an oxidant for TPNH. However, when  $C^{14}O_2$  is incorporated into isocitrate or  $C^{14}$ -isocitrate is converted to  $\alpha$ -ketoglutarate, an oxalosuccinate pool acquires negligible activity suggesting that if oxalosuccinate is the intermediate, it must be tightly bound. The mechanisms of isocitric dehydrogenase and aconitase have been investigated by Englard & Colowick (226). When *cis*-aconitate was converted to  $\alpha$ -ketoglutarate by the two enzymes in the presence of  $D_2O$ , no D was incorporated into the TPNH. This result fits the idea that the  $\alpha$ -H of isocitrate is transferred to  $TPN^+$ . In the same experiments (226) aconitase is shown to be stereospecific, with respect to H, i.e., during

the series of *cis*-aconitate—citrate—*cis*-aconitate, the same H introduced into citrate is removed on reconversion to *cis*-aconitate. The studies also support the hypothesis of Speyer & Dickman (227) that the aconitase reaction involves a carbonium ion rather than *cis*-aconitate per se as an intermediate. The role of *trans*-aconitate in the tricarboxylic acid cycle is the subject of a preliminary report (228) in which *trans*-aconitate was shown to act as a non-competitive inhibitor for the oxidation of the tricarboxylic acid, with the point of inhibition apparently isocitric dehydrogenase rather than aconitase.

Kidney homogenates from rats on a vitamin D deficient diet show a greater ability to oxidize citrate than homogenates from control animals (229). Liver homogenates did not show such an effect. In later studies using kidney mitochondria, an *in vitro* inhibition of citrate and isocitrate by vitamin D has been shown (230) although other substrates with the possible exception of glutamate are not affected.

Peters & Wakelin (231) have found that the synthesis of fluorocitrate from oxalacetate and fluoroacetate in kidney particles is inhibited by the presence of acetate, suggesting that fluoroacetate and acetate are competitive in the activation pathway for fluoroacetate. Watland *et al.* (232) have studied the inhibitory effects of the ethyl esters of difluoroacetoacetate, fluoro-oxalacetate and fluoroacetate on the formation of citrate by kidney mitochondria and also suggest competition of the fluoroacetate compounds with acetate.

In a preliminary report, Smith *et al.* (233) describe the separation of the *E. coli* succinate activation system into two parts:



25.



The first of these enzymes, thiokinase, was measured by determining the ADP produced by use of pyruvic kinase and lactic dehydrogenase, although  $\text{CoA-S} \sim \text{P}$  was also detected and its properties described. The second enzyme, phosphoryl CoA transferase, was purified some hundredfold.

Malic dehydrogenase from ox heart mitochondria has been highly purified and its properties and specificity described (234). The enzyme can oxidize oxaloglycollate, D(-) and meso-tartrate, tartronate, and  $\alpha$ -OH glutamate in addition to L-malate although the latter is oxidized more rapidly than any of the other substrates. Resting *E. coli* cells show adaptive ability to ferment D-malate (235) although the nature of the reaction is not understood.

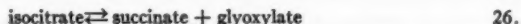
Saz & Hubbard (236) have described the purification of malic enzyme from the muscle of the roundworm, *ascaris*. Unlike previously described malic enzymes from liver (237) and lactobacillus (238), the *ascaris* enzyme does not decarboxylate oxalacetate and is active with both DPN and TPN. The nucleotide specificity of oxalacetic carboxylase has been shown to be limited to guanosine and inosine polyphosphates (239). The succinate requirement of a mutant of *Neurospora crassa* is apparently due to a lack of

oxalacetic carboxylase in the mutant (240). From studies with the leaves of the succulent plant *kalanchoe*, Walker (241, 242) has proposed that phosphopyruvate carboxylase (243) is responsible for the dark fixation of  $\text{CO}_2$  in these plants, and a similar proposal has been made by Moyse & Jolchine (244) on the basis of experiments with bryophyllum.

#### METABOLIC SYSTEMS RELATED TO THE TRICARBOXYLIC ACID CYCLE

The metabolism of growing numbers of compounds can be related to the tricarboxylic acid cycle. In some cases, the reactions provide alternative pathways for part of the tricarboxylic acid cycle while in others the compounds are linked with the cycle through intermediates.

The "glyoxylate shunt."—Campbell *et al.* (245) first reported the formation of glyoxylate and succinate from tricarboxylic acids in extracts of *Pseudomonas aeruginosa*. The substrate was reported to be *cis*-aconitate or citrate but later reports make it probable that the true substrate is isocitrate.



The isocitratase (isocitric lyase) reaction has been further studied in *Pseudomonas* (246, 247), in various mold species (248) and in *E. coli* (249). As indicated the reaction is believed to be reversible, at least under certain conditions (245, 247, 248).

More recently, Wong & Ajl (250) reported another interesting reaction in *E. coli* involving glyoxylate, the malate synthetase reaction shown below:



During the past few months, the latter reaction has also been demonstrated in *Pseudomonas* by Kornberg & Madsen (251) with glyoxylate replaceable by isocitrate. The combination of the above two reactions with part of the tricarboxylic acid cycle has been proposed as a mechanism for the formation of dicarboxylic acids from acetate (251, 252, 253). The reactions involved can be written as shown in Figure 5:

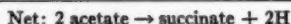
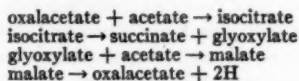


Fig. 5. The synthesis of succinate by the "glyoxylate" shunt (Reaction 28).

When isocitratase is the sole source of glyoxylate as in the above series, the first part of the tricarboxylic acid cycle must be operating at a rate at least equal to the overall rate of succinate formation. It has been pointed out (251, 252) that these reactions could account for the accumulation of citrate (254) and fumarate (255) in molds and for the synthesis of various materials derived from di- and tricarboxylic acids in organisms growing on a two-carbon substrate. The net synthesis of a tricarboxylic acid will require in

addition to the reactions illustrated in Figure 5 the conversion of succinate to oxalacetate and condensation of the latter with a third molecule of acetate. It is important to note that thus far, the malate synthetase reaction has been demonstrated only in microorganisms adapted to growth on acetate (250, 251) and in the castor bean (256). In the latter isocitritase and malate synthetase have been demonstrated and hypothesized to serve as a mechanism for the conversion of fat to carbohydrate. Callely *et al.* (257) note the presence of isocitritase and malate synthetase in a vibrio and a role of glyoxylate in the oxidation of fatty acid is suggested in this organism. The isocitritase content of this organism was increased tenfold by growth on acetate.

These very interesting developments raise several questions among which the foremost is the applicability of the mechanism to higher organisms for the transformation of fatty acids to carbohydrates. At present the two key enzymes, isocitritase and malate synthetase, have not been demonstrated in animals and there is evidence to suggest that at least one of these enzymes is adaptive.

Glyoxylate oxidation has been demonstrated in plants (258) and pseudomonas (259) and the metabolism of glyoxylate has also been a subject of study in animals (260, 261) where glyoxylate plays a role in glycine metabolism and perhaps can be further oxidized. It is interesting to note that glyoxylate has been reported to inhibit respiration of liver homogenates (262) and to cause the accumulation of citrate from oxalacetate (263). However, citrate oxidation was not inhibited by glyoxylate leading to the hypothesis that glyoxylate reacted with oxalacetate to form an inhibitor of citrate oxidation. The results might also be interpreted as a reversal of the isocitritase reaction to form a tricarboxylic acid, although this seems unlikely. The reversal of the isocitritase reaction is not yet certain since it has been reported to occur in some types of preparations and not in others (246, 247). It is possible that reaction at least in the reverse direction is more complicated than written. Isocitritase has been recently purified eightyfold from baker's yeast (264) but the preliminary report does not deal with reversibility. Little

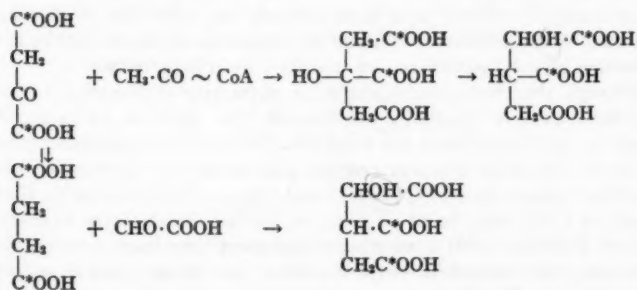
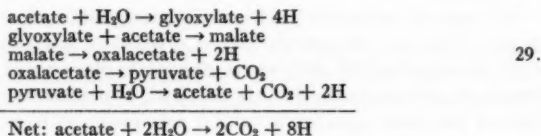


FIG. 6. Variations in labeling of isocitrate formed by different pathways.

tracer work has been carried out with the isocitritase reaction but it would appear probable that the labeling of isocitrate produced by this pathway would be different than that produced via the condensation and aconitase reactions.

With succinate-1-C<sup>14</sup> and acetate-C<sup>13</sup> as substrates the tricarboxylic acid cycle will never lead to labeling in the primary —COOH distal to the —CHOH— group. However, reversal of the isocitritase reaction with succinate-1-C<sup>14</sup> and glyoxylate will lead to labeling of the lower primary —COOH and eventually also to labeling in the other primary —COOH as glyoxylate acquires labeling from isocitrate formed by the tricarboxylic acid pathway. If the lower mechanism is valid it should be possible to detect the presence of isocitritase by the degradation of isocitrate formed from dicarboxylic acids labeled as shown.

The source of glyoxylate formation is interesting. If glyoxylate can be formed from acetate, then a true dicarboxylic oxidative cycle can exist as shown below:



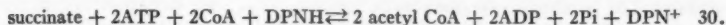
The oxidation of acetate by yeast to CO<sub>2</sub> via glycollate, glyoxylate, and formate has been reported by Bolcato *et al.* (265) by use of trapping techniques with labeled substrates. Glyoxylate pools received labeling from acetate but the dilution was rather high and a pathway involving isocitritase cannot be excluded.

There are other possible sources for glyoxylate. In *Pseudomonas* (266) glycine has been shown to be converted to glyoxylate and the oxidation of glycine is stimulated by succinate. It is proposed that isocitrate is formed via isocitritase from glyoxylate with subsequent oxidation to isocitrate to succinate by the usual pathway. One other possible source of glyoxylate should be mentioned. In chloroplasts from spinach, the oxidation of glycolate is stimulated by illumination (267) and the suggestion is made that in normal metabolism glycolyl substrates are provided by transketolase.

Although the glyoxylate shunt is an attractive explanation for many metabolic events it should be emphasized that thus far no quantitative evaluations of this pathway are available. Additional alternative pathways such as the succinate cleavage enzyme (see below) or the reaction of two glyoxylates to form hydroxypyruvate and CO<sub>2</sub> as demonstrated by Krakow & Barkulis (268) may be of as great or greater quantitative importance. Glasky & Rafelson (269) have studied the short time incorporation of C<sup>14</sup>-acetate into the intracellular organic acids of acetate adapted *E. coli* in log phase of growth. The first substances labeled were succinate and glutamate while malate and isocitrate did not become labeled in the early period. These

preliminary results do not seem to fit the glyoxylate shunt pathway.

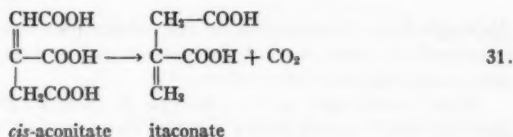
*Succinate cleavage.*—Seaman (270) has presented a more complete report of his studies on the succinate cleavage enzyme of *Tetrahymena pyriformis*:



The enzyme has been purified about one hundred fifty-fold, shows almost absolute requirements for the various substrates and the stoichiometry for succinate, acetyl CoA (as acethydroxamic acid), ATP, ADP, Pi and  $\text{DPN}^+$  lends strong support to the above mechanism. Citrate can be formed in the presence of oxalacetate and condensing enzyme. The possibility that succinate can be converted to acetate via oxalacetate and pyruvate has been ruled out by experiments with succinate-1- and -2- $\text{C}^{14}$  in which the labeling in acetate is found in the  $-\text{COOH}$  and  $-\text{CH}_3$  groups respectively. Although the evidence supporting the proposed mechanism is strong and the purification of the enzyme achieved is considerable, it is a little difficult to reconcile the multiplicity of events required in this mechanism with the action of a single enzyme.

The reaction is reported to be somewhat reversible although the evidence is less extensive than for the cleavage reaction. This enzyme acting in the reverse direction could synthesize dicarboxylic acids from acetate and hence carbohydrate from fatty acid. Preliminary studies indicate that the enzyme is also present in *E. coli* and in various rat tissues (270). The assay consisted of the production of acethydroxamic acid from succinate in the presence of malonate and arsenite to inhibit the usual succinate-acetate pathways. These observations if confirmed by further purification or succinate- $\text{C}^{14}$  studies would be of great interest. It should be noted that the studies of Swim & Krampitz (271) on the formation of succinate from  $\text{C}^{13}$ -acetate in *E. coli* showed no indication of the above reaction (in the condensation direction).

*Itaconate and mesaconate metabolism.*—Itaconate (methylene succinate) has long been known as a product of the metabolism of certain molds. Bentley & Thiessen have carried out a series of studies (272, 273, 274) on the mechanism of synthesis of this substance in *Aspergillus terreus* and have come to the conclusion that it results from the decarboxylation of *cis*-aconitate as shown below:



The labeling of itaconate formed from glucose-1- $\text{C}^{14}$ , acetate-1- $\text{C}^{14}$  and succinate-1- or -2- $\text{C}^{14}$  supports the idea that the  $\text{CO}_2$  comes from a primary carboxyl, presumably the  $-\text{COOH}$  arising from the acetate  $-\text{COOH}$  in the condensation reaction.

Itaconate may not be limited to a role as a by-product of mold metabolism since Adler (275) has recently reported that itaconate is oxidized by guinea pig liver mitochondria at a rate comparable to better known substrates. The products are glutamate, lactate,  $\text{CO}_2$ , and mesaconate (methyl fumarate). There is strong evidence that the first step of itaconate metabolism involves the formation of a CoA derivative but the further reactions are not clear. One possible pathway could involve  $\text{CO}_2$  fixation to *cis*-aconitate, the reversal of the mold decarboxylation mentioned above.  $\text{CO}_2$  is fixed into citrate and into the  $\alpha$ -COOH of glutamate in the presence of itaconate. The position in the latter compound is in accord with fixation in oxalacetate or malate and formation of citrate via the condensation reaction rather than by a reversal of the *cis*-aconitate decarboxylation reaction. A second possible pathway for the metabolism of itaconyl CoA could be via mesaconate and citramalate as shown by Barker *et al.* (276) in the fermentation of glutamate by *Clostridium tetanomorphum*. Their studies indicate that all of the reactions are reversible and that the  $-\text{CH}_3$  group of mesaconate arises from C-3 of glutamate.

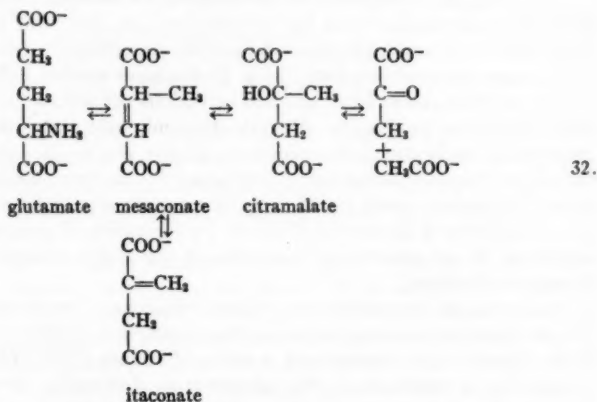


FIG. 7. Conversion of glutamate and itaconate to pyruvate and acetate.

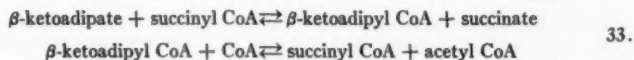
Although liver mitochondria do not oxidize mesaconate or citramalate, the intermediates may be CoA derivatives and activating enzymes for these compounds may not be present.

*Other dicarboxylic acids.*—Koepe & Hobbs (277) have administered glutarate-3- $\text{C}^{14}$  to the intact rat and have studied the  $\text{C}^{14}$  distribution in glutamate, aspartate and alanine in an attempt to determine whether glutarate is converted directly to  $\alpha$ -ketoglutarate or is cleaved to acetate. The results support the latter pathway since virtually all of the labeling appears in the  $-\text{COOH}$  groups of the above amino acids. The conversion of 3-C of glutarate to the carboxyl of acetate might suggest  $\beta$ -oxidation followed by



cleavage to acetate and malonate with conversion of the latter to acetate as suggested by Nakada *et al.* (278) in experiments with slices of various rat tissues.

Katagiri & Hayaishi (279) have studied the metabolism of  $\beta$ -ketoadipic acid in *Pseudomonas fluorescens* and propose the following pathway:



The first enzyme has been purified thirtyfold and found to be specific for succinyl CoA.  $\beta$ -Ketoadipyl CoA has been prepared and studied in the second reaction. As indicated both reactions appear to be reversible. Oxalate decarboxylation has been studied in the fungus, *Collyria velutipes* (280). An enzyme has been highly purified which converts oxalate to  $\text{CO}_2$  and formate. This mechanism for oxalate decarboxylation differs from that shown previously in neurospora and torula (281) or from the reaction in higher plants (282). The metabolism of oxalate- $\text{C}^{14}$  in animals has been studied by Just & Bernhard (283, 284) although no conclusions as to mechanism have been reached.

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## WATER-SOLUBLE VITAMINS, PART I<sup>1</sup> (FOLIC ACID, B<sub>12</sub> GROUP, CHOLINE)

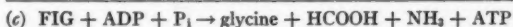
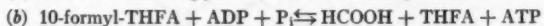
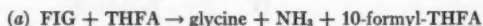
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The papers that have been selected for this review were those that were available to the writer from about December 1, 1956, to mid-October, 1957. During this period hundreds of papers have appeared bearing on the chemical, biochemical, microbiological, nutritional, physiological, and clinical aspects of these vitamins. The reviewer has endeavored to select a representative group of these publications to illustrate biochemical progress in this field.

### FOLIC ACID AND RELATED COMPOUNDS

*Formimino and formyl group transfer.*—Advances in knowledge of the intermediary metabolism of the purines, pyrimidines, and amino acids have given a clearer understanding of the chemical nature of the coenzymatic forms of folic acid, their interrelationships, and the manner in which they function. This is particularly well illustrated by the biochemical events leading to the present concept of  $-\text{CH}=\text{NH}$  (formimino) group transfer mediated by folic acid coenzymes. Studies of the enzymic degradation of xanthine by extracts of *Clostridium cylindrosporum* led Rabinowitz & Pricer (1) to identify formiminoglycine as a metabolic product. The subsequent finding that THFA was required for the catabolism of FIG (2, 3) led to the suggestion (2) for a role of THFA in formimino group transfer. The reactions involved in the over-all degradation of FIG were shown by Rabinowitz & Pricer (4) to require ADP and P<sub>i</sub> and to give glycine, ammonia, and formate with the concomitant generation of a mole of ATP as shown in equation (c). As reported in last year's review (5),



reaction (c) was shown to occur in a stepwise fashion through the participa-

<sup>1</sup> The following abbreviations are used in this chapter: ADP and ATP for adenosine diphosphate and adenosine triphosphate, respectively; AICAR for 4-amino-5-imidazolecarboxamide; AMP for adenosine monophosphate; CF for citrovorum factor or 5-formyltetrahydropteroylglutamic acid; DNA for deoxyribonucleic acid; DPN and TPN for diphosphopyridine nucleotide and triphosphopyridine nucleotide, respectively; FIG for formiminoglycine; FIGLU for formiminoglutamic acid; N.M.M. for N-methylnicotinamide; PABA for *p*-aminobenzoic acid; PGA for pteroylglutamic acid; P<sub>i</sub> for inorganic phosphate; RNA for ribonucleic acid; and THFA for tetrahydropteroylglutamic acid.

tion of folic acid coenzymes as given by equations (a) and (b). Rabinowitz & Pricer have now demonstrated (6) that reaction (a) is the sum of three reactions as shown in Figure 1.

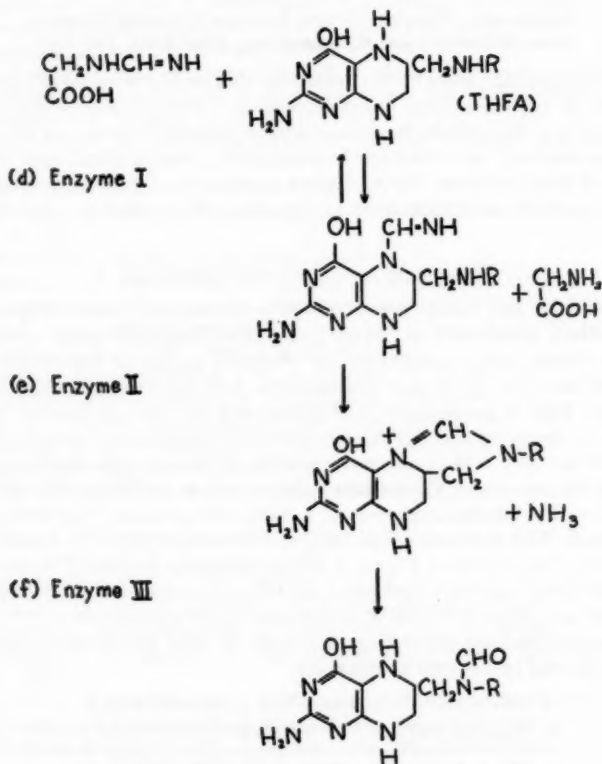
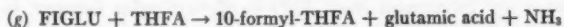


FIG. 1. Mechanism of formimino group transfer according to Rabinowitz & Pricer (6).

Enzymes I and II, reactions (d) and (e), were satisfactorily separated and purified about tenfold from crude extracts of *Cl. cylindrosporium* so that detailed studies of these reactions were possible. Enzyme I, termed FIG formimino transferase, catalyzed the transfer of the formimino group of FIG to THFA to give formimino-THFA and glycine. Sufficient THFA-reaction product was isolated from the reaction mixture to permit chemical and biological study (7). The intermediate had these properties in common

with 5-formyl-THFA: identical absorption maxima in the ultraviolet, conversion of 5,10-methenyl-THFA (anhydroleuovorin) by the action of dilute acid, similar stability to oxygen, had twice the microbiological activity of synthetic leuovorin for *Pediococcus cerevisiae*; from these and other considerations the intermediate was judged to be a single diastereoisomer of 5-formimino-THFA. This derivative of THFA, but not 5-formyl-THFA, was converted by enzyme II, formimino-THF cyclodeaminase, to 5,10-methenyl-THFA and ammonia, reaction (e); 5,10-methenyl-THFA was then converted in turn to 10-formyl-THFA by enzyme III, methenyl-THF cyclohydrolase, reaction (f).

Much is now known about the pathway of histidine degradation in various species [cf. (8) for discussion] and certain of these studies with mammalian enzyme systems have also revealed a formimino transfer step involving THFA which independently corroborates the mechanism of formimino transfer described above. Borek & Waelsch (9) had earlier demonstrated the formation of formiminoglutamic acid (formamidinoglutamic acid) as a product of the action of cat liver homogenates of urocanic acid arising from histidine. The enzyme urocanase has since been purified and the reaction mechanism studied in detail by Miller & Waelsch (10). These workers have now published two papers (11, 12) dealing with the enzymic degradation of FIGLU which support earlier brief reports from their laboratory (13, 14) for a role of FIGLU as a formylating agent. In a suitably purified enzyme system from rat or calf liver, FIGLU was shown to react with PGA to give 10-formyl-PGA in essentially quantitative yield. The requirements for this reaction were a reducing agent and an activator, the latter of which could be met by certain tricarboxylic acids, TPNH or reduced PGA. It was visualized that catalytic amounts of THFA were formed in the system which then reacted with FIGLU forming 10-formyl-THFA which in turn was converted to 10-formyl-PGA either by oxidation or by transformylation to PGA, THFA being regenerated to repeat the cycle. Rauen (15) has also reported that with homogenates of rat or pig liver 10-formyl-PGA was formed when PGA was incubated under certain conditions with histidine or other metabolites known to furnish the 1-carbon unit. Perhaps purification of this latter system will show a mechanism for the synthesis of 10-formyl-PGA similar to that proposed above (11). In a second paper Miller & Waelsch (12) discussed in detail the mechanism of the formimino transfer from FIGLU to THFA by soluble liver extracts. The over-all reaction at a neutral pH was considered to be [compare with reaction (a)]:



When FIGLU was incubated with substrate quantities of THFA and the reaction mixture was acidified, 5,10-methenyl-THFA was concluded to be a reaction product on the basis of ultraviolet spectrum. In support of over-all reaction (g) it was shown that equimolar quantities of 5,10-methenyl-THFA, glutamic acid, and ammonia were formed. It was considered that reaction

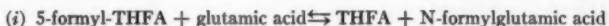
(g) proceeded stepwise through a reversible formimino transfer as shown by equation (h).



As evidence for this postulate it was shown that the addition of glutamic acid to the system decreased formylation of THFA, and that the addition of C<sup>14</sup>-glutamic acid led to radioactivity in FIGLU. The specificity of the formyl donor to THFA, reaction (h), was studied and when formylglutamic acid was substrate, a product was formed which was identified as 5-formyl-THFA. This was taken as evidence that in the formimino transfer reaction (h) the formimino group is first attached to the 5-nitrogen atom of THFA. Miller & Waelsch proposed that 5-formimino-THFA underwent a loss of ammonia and rearrangement to 5,10-methenyl-THFA, the latter of which may then be hydrolyzed to 10-formyl-THFA, this sequence of events being in accord with reactions (d, e, f), the only difference being that in this latter instance the initial substrate was FIGLU rather than FIG. Tabor & Rabinowitz (16) have also reported briefly that rabbit liver contains three enzymes which catalyze reaction (h) via the mechanism of reactions (d, e, f). Thus these studies of FIG and FIGLU degradation have led to the recognition of the probable existence of a new folic acid coenzyme, 5-formimino-THFA, and have established an enzymatic pathway for the biosynthesis of 5,10-methenyl-THFA and 10-formyl-THFA which may then function in reactions of 1-carbon metabolism at the formate level of oxidation. The origin of 10-formyl-THFA via the above route from the catabolism of histidine may well be an important manner by which the 1-carbon unit becomes available for *de novo* synthesis in mammals. Indirect evidence in support of this view was obtained by Broquist and Luhby (17) who reported that when children with acute leukemia were treated with 4-amino-10-methyl folic acid (Amethopterin), a folic acid antagonist, FIGLU was excreted in the urine. Since Amethopterin is known to block at a stage in the reduction of folic acid to THFA it can be seen, reaction (d), that if THFA is limiting, a formimino amino acid might be expected to accumulate.

The biological significance of CF has been somewhat of an enigma. Its occurrence in nature and its biosynthesis in a number of enzyme systems from folic acid, HCOOH and a reducing agent might be explained on the basis of a chemical rearrangement from 10-formyl-THFA formed enzymatically (18). Moreover 5-formyl-THFA has not been shown to be active per se in an isolated enzyme system without prior activation by ATP. Thus a recent paper by Silverman *et al.* (19) is of particular interest in that an enzyme system is described in which CF directly participates without the aid of ATP and moreover 5,10-methenyl-THFA or 10-formyl-THFA were not involved. Keresztesy & Silverman (20) had earlier observed that when CF was incubated with a hog liver fraction, a loss of CF activity resulted with the simultaneous formation of an arylamine. Hog liver has now been found to contain an enzyme that catalyzes the transfer of the formyl group of CF to

glutamic acid, giving formylglutamic acid and THFA as shown in equation (i).



Reaction (i) is readily reversible (19), thus providing a mechanism for the synthesis of CF. Miller & Waelsch (12) had also observed CF formation from formylglutamic acid incidental to their study of formimino transfer between FIGLU and THFA. THFA, a product of the forward reaction (i) has previously been shown to undergo non-enzymatic oxidation to *p*-amino-benzoyl glutamic acid and uncharacterized pteridine(s) (cf. last year's review for discussion); details of this latter aspect of the problem have now appeared in papers by Futterman & Silverman (21) and Dinning and co-workers (22). Blakley (23) has made a careful study of the chemical properties of dihydro- and tetrahydrofolic acid including their lability to air oxidation and has identified xanthopterin and 2-amino-4-hydroxy-6-methylpteridine as oxidation products.

*Serine and glycine interconversion.*—A number of papers have appeared dealing with the nature of the folic acid derivative concerned in the serine-glycine interconversion. It is clear from previous investigations that the folic acid intermediate carrying the 1-carbon unit is at the oxidation level of formaldehyde. Blakley has presented additional evidence (23) supporting his earlier postulate (24) for the intermediate as structure (I), Figure 2, in that, when dihydro- or tetrahydrofolic acid were prepared chemically, they retained most coenzyme activity during aeration when allowed to react with formaldehyde. Substance (I) may be viewed as the anhydride of hydroxymethyl-THFA; however attempts to demonstrate the existence of such a type intermediate which could be converted enzymatically into the  $\beta$ -carbon of serine or formaldehyde were inconclusive (25). Kisliuk (26) has also studied the non-enzymatic reaction between formaldehyde and THFA, Reaction (j), Figure 2, and showed that both the 5-N and 10-N atom of THFA must be free to bind HCHO chemically and to participate in enzymatic formation of serine, thus giving further support for structure (I). He speculates that substance (I) formed chemically may react enzymatically

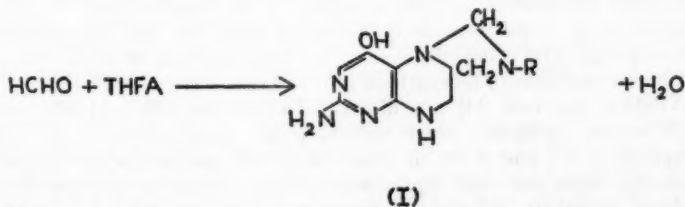
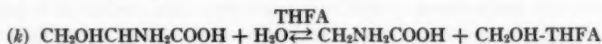


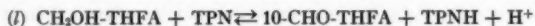
FIG. 2. Non-enzymatic reaction of formaldehyde and tetrahydrofolic acid after Kisliuk (26).

with glycine to give an N<sup>5</sup>-alanyl-THFA which could be hydrolyzed to serine and THFA. A THFA-mediated reaction between glycine and HCHO by extracts of *Clostridium acidi-urici* was reported by Beck, Sagers & Morris (27); the reaction product was pyruvate which was assumed to arise via serine.

The cofactor requirements of the serine hydroxymethylase enzyme system, reaction (k), of beef liver have been carefully studied by Huennekens, Hatefi & Kay (28).



The complete system was shown (28) to require DPN, TPN, reduced glutathione, pyridoxal phosphate, THFA and a partial dependence for Mn<sup>++</sup>. A later paper by this group (29) describes the purification of the enzyme, hydroxymethyl-THFA-dehydrogenase, and the participation of TPN in the oxidation of hydroxymethyl-THFA generated in reaction (k), to 10-formyl-THFA, reaction (l).



Reaction (l) is readily reversible. Huennekens (30) has reported briefly that beef liver also contains an enzyme system capable of deacylating 10-formyl-THFA to THFA and HCOOH; superficially this reaction is similar to that described for *Clostridium kluyveri* (4), reaction (b).

Peters & Greenberg (31) have continued earlier studies from Greenberg's laboratory on the mechanism of the conversion of CF to a coenzyme form participating in serine synthesis from glycine by sheep liver enzymes. They visualize the activation of CF by ATP through an enzyme-AMP-PP complex giving rise, perhaps via 5,10-methenyl-THFA, to 10-formyl-THFA. The latter is then reduced by TPNH to 10-hydroxymethyl-THFA in accordance with reverse reaction (l) and can then react with glycine giving serine, reverse reaction (k). The importance of reaction (k) in 1-carbon metabolism is made clear from quantitative studies of serine metabolism in rat liver slices by Elwyn *et al.* (32) which suggest that reaction (k) is the major reaction of serine in the liver. Thus hydroxymethyl-THFA arising from serine degradation provides a source of the 1-carbon unit for biosynthetic work at the formaldehyde level of oxidation, and the enzyme, hydroxymethyl-THFA-dehydrogenase, can then function to make the 1-carbon unit available for biosynthesis at the formate level of oxidation.

Wright & Anderson (33) have discovered an enzyme, folic acid reductase in *Clostridium sticklandii* which reduces either pteroylglutamic acid or pteroyltriglutamic acid to the dihydro level of reduction; serine functions as the electron donor and other requirements of the system are orthophosphate, pyridoxal phosphate, 2,3 dimercaptopropanol, and coenzyme-A. The authors indicate that dihydropteroyltriglutamic acid may be an intermediate in the pteridine transformations accompanying the conversion of serine to



glycine by this organism, although glycine has not as yet been identified as a product of the reaction.

*Folic acid and thymine synthesis.*—The early observation that the requirement of certain lactic acid bacteria for folic acid could be met under certain conditions by thymine, led to the view that folic acid was concerned in thymine synthesis. Ultimately it was established that the 5-methyl group of thymine originated from a 1-carbon unit by a process involving folic acid; but it has only been recently that work on the biosynthesis of pyrimidines has advanced to the point where the enzymatic step involving folic acid has emerged. Earlier work of Friedkin & Roberts (34) had established that deoxyuridine was a precursor of thymidine in an enzyme system inhibited by Aminopterin or augmented by CF. Friedkin reported briefly (35) on an enzyme system of *Escherichia coli* that converts deoxyuridine-5'-phosphate to thymidylic acid in the presence of THFA, ATP,  $Mg^{++}$ . Serine or formaldehyde could serve as 1-carbon donors, but could be replaced, together with THFA, by hydroxymethyl-THFA. The system contains a kinase which rapidly converts thymidylic acid to thymidinetriphosphate, so that the latter was the major product in the reaction. In a similar system from thymus gland Phear & Greenberg (36) studied the methylation of deoxyuridine by formaldehyde to give principally thymidylic acid. The cofactor requirements were the same as those in Friedkin's system but in addition DPNH was required which led Phear and Greenberg to suggest the involvement of DPNH in the reduction of the 5-hydroxymethyl derivative of the nucleotide to the methyl group.

*Methionine, folic acid relationships.*—Doctor *et al.* (37) have studied the incorporation of  $C^{14}$  from serine-3- $C^{14}$  or formaldehyde- $C^{14}$  into methionine by an enzyme system in chick liver acetone powder. They observed that incorporation was stimulated by PGA, THFA, and homocysteine, and proposed that hydroxymethyl-THFA may condense with homocysteine to give an intermediate in methionine biosynthesis. Vitamin  $B_{12}$  had no effect on methionine synthesis in this system (38). Stekol and co-workers (39) presented new evidence that deficiencies of folic acid or vitamin  $B_{12}$  in chicks did not impair transmethylation reactions either to or from methionine. They could find no evidence for an involvement of vitamin  $B_{12}$  in the reduction of homocystine to homocysteine *in vivo*.

Several reports have appeared on the origin of  $C_{28}$  in ergosterol in yeast. Although formate can be incorporated into this position (40, 41), it appears more likely from reports of Alexander *et al.* (41, 42) that the methyl group of methionine serves directly in a transmethylation reaction to the ergosterol hydrocarbon structure to furnish  $C_{28}$ . Any effect of folic acid on ergosterol synthesis (41, 42) appears, then, to be an indirect one by functioning in the *de novo* synthesis of the  $S-CH_3$  group of methionine via homocysteine and the 1-carbon unit.

*Folic acid and histidine synthesis.*—Using the technique of inhibition analysis, Broquist (43) described conditions wherein the toxicity of 4-amino-

PGA (Aminopterin) for growth of *Torula cremoris* could be reversed non-competitively by histidine. This effect could not be shown in the absence of adenine, thus emphasizing previously demonstrated metabolic relationships between purines and histidine and implying the involvement of folic acid in histidine biosynthesis. These findings become more significant from a communication by Moyed & Magasanik (44) who present evidence for a catalytic role of the purine nucleotides in the biosynthesis of histidine. These workers found that when extracts of *Salmonella typhimurium* were incubated with AMP, ribose-5-phosphate, ATP, and glutamine, the products of the reaction were AICAR and imidazoleglycerolphosphate, the latter of which is a recognized precursor of histidine (45). The postulated role of folic acid in histidine synthesis in *T. cremoris* (43) could be an indirect one concerned in the regeneration of AMP from AICAR.

*Biosynthesis of folic acid; pteridine conversions.*—Korte & Barkemeyer (46) have described the synthesis on a microscale of 2,4,5-triamino-6-hydroxypyrimidine, xanthopterin, and isoxanthopterin in which C-5 of the pyrimidine and C-4 of the pteridines are labelled with  $C^{14}$ , thus permitting a study of their possible incorporation into folic acid. In a subsequent note, Korte and co-workers (47) state that certain microorganisms converted 4- $C^{14}$ -xanthopterin and 4- $C^{14}$ -isoxanthopterin into folic acid and that these microorganisms could also degrade these pteridines to uracil; details of this work will be awaited with great interest. Weygand *et al.* (48) studied the uptake of PABA (carboxyl  $C^{14}$ ) and PGA-2- $C^{14}$  by various strains of bacteria and observed that these compounds but not 2,4,5-triamino-6-hydroxypyrimidine or certain pteridines were incorporated into "bound folic acid" of *Enterococcus stei*. Evidence for the occurrence of a  $\gamma$ -linked triglutamate derivative of CF in cells of *Bacillus subtilis* has been presented by Hakala & Welch (49); *Streptococcus faecalis* could not utilize this derivative for growth, but enzymes capable of its synthesis were found in the cell. Koft & Morrison (50) have described some interesting experiments wherein two bacterial cultures were chosen which required folic acid and PABA respectively for growth, but the organisms could grow satisfactorily in mixed culture in the absence of these metabolites; such results suggest one approach to the route of biosynthesis of folic acid. Nurmikko stressed the potential usefulness of symbiosis techniques in studying biochemical pathways (51), and described in detail appropriate apparatus for such experiments (52).

Forrest, Glassman & Mitchell (53) have demonstrated the conversion of 2-amino-4-hydroxypteridine to isoxanthopterin by extracts of drosophila and noted the similarity of the converting enzyme to xanthine oxidase in cream. Forrest and co-workers (54) noted that when *Anacystis nidulans*, a blue-green algae, was briefly exposed to a cold temperature, a loss of photosynthetic activity resulted and a blue fluorescent material appeared which proved to be principally 2-amino-4-hydroxy-6-carboxypteridine.

*Nutritional studies in animals.*—Loss of pigmentation of the feathers of young turkeys on low lysine diets is well recognized as a result of lysine

deficiency. Klain, Hill & Branion (55) have observed similar but less severe effects in chicks on low folic acid diets and suggest that both lysine and folic acid may have a role in feather pigmentation. Further work has appeared on the toxicity of glycine for chicks; Kratzer & Lantz (56) noted that on a low folic acid diet the addition of 2 to 4 per cent glycine resulted in depressed growth, increased mortality, and cervical paralysis. These effects were not duplicated with equimolar quantities of DL-serine, but the effects were counteracted by increasing the level of folic acid in the diet. Misra, Woodruff & Darby (57) studied the effect of dietary intake of ascorbic acid and folic acid in monkeys on the level of these substances and CF in the liver. The level of folic acid intake did not influence the ascorbic acid content of the liver, but ascorbic acid markedly enhanced the content of CF in monkeys receiving folic acid, in agreement with similar observations from numerous enzyme studies. An excellent paper by Nelson (58) has appeared which contains considerable illustrative material summarizing work of her group concerning the production of congenital malformations in the rat fetus as a consequence of folic acid deficiency in the mother. Haas *et al.* (59) made the interesting observation that mice on a diet low in folic acid or receiving Amethopterin were spared from a fatal dose of lymphocytic choriomeningitis. The effect of the folic acid deficiency appeared not to be directed *per se* against the virus, for it was recovered from the brains of protected mice in amounts comparable to those in the controls.

*Method of assay.*—A tabulation of the fluorescence characteristics of a wide number of biological constituents, including folic acid and CF, has appeared in a paper by Duggan *et al.* (60). It is suggested that the application of spectrophotofluorometry be given consideration as an analytical tool for these metabolites. Present microbiological methods for folic acid assay are admittedly not satisfactory (61); further research in this difficult area is needed.

#### FOLIC ACID ANTAGONISTS

*Clinical aspects.*—Interest in the 4-aminofolic acid antagonists stems chiefly from their usefulness, albeit limited, in the treatment of acute leukemia. The proceedings of an international symposium on leukemia has been published recently (62) and contains considerable material, some of which has not appeared previously, concerning folic acid metabolism in relation to leukemia. Ellison & Hutchison (62a), confirmed and extended an earlier observation of Swendseid *et al.* (63) that the CF content of leukocytes from individuals with acute leukemia was significantly higher than in leukocytes of normal individuals. These findings may be correlated with studies by Winzler *et al.* (62b, 64) who found that the *in vitro* incorporation of formate- $C^{14}$  into human leukocytes from individuals with acute leukemia was greater than into normal cells, and that the incorporation was stimulated by leucovorin and not by folic acid. From these observations one might expect the leukemic cell to be somewhat more vulnerable to the action of a folic acid antagonist than a normal cell. Indeed, Winzler and co-workers demon-

strated that formate incorporation into acute leukemic cells could be blocked by Amethopterin, but the antagonist had little or no effect on incorporation by normal leukocytes. The authors speculate that these differences in sensitivity to Amethopterin might be explained on the basis of differences in CF synthesis, permeability to the antagonist, affinity of the antagonist for certain 1-carbon transferring enzyme systems, or an alternative metabolic pathway of 1-carbon metabolism. Such arguments might also explain in part the variable clinical response of children with acute leukemia to the folic acid antagonists. Hutchison (62c) has summarized much of her data on metabolic differences between cultures of *S. faecalis* sensitive and resistant to Amethopterin; additional discussion on resistance to folic acid antagonists is also given by Nichol (62d). This latter paper presents an *in vitro* test system in which the degree of inhibition by Amethopterin of CF synthesis from folic acid by various lines of leukemic cells can be studied. Such studies may ultimately yield important information bearing on effectiveness of treatment of acute leukemia with Amethopterin. Clinical aspects of this subject were comprehensively discussed at this symposium (62) and elsewhere by Farber *et al.* (65), and Burchenal *et al.* (66).

An excellent review on the clinical use of combinations of drugs including the folic acid antagonists in cancer chemotherapy has appeared (67). Goodwin has discussed rather striking examples of synergistic effects between the sulfonamides and pyrimethamine (5-*p*-chlorophenoxy-2,4-diamino-6-ethylpyrimidine), a folic acid antagonist, in malaria and toxoplasmosis (68). In the latter instance, side reactions such as thrombocytopenia and leucopenia may develop which have been reported to be controlled by the simultaneous administration of CF (69); it was argued that toxoplasma, in contrast to man, cannot utilize CF, and therefore CF exerted a favorable selective action against the toxicity of these antimetabolites in the host. Such findings emphasize the important bearing the nutritional requirements of host and parasite may have on the chemotherapeutic treatment.

*Mechanism of action.*—Evidence for the lack of selective action of Amethopterin towards tumor tissue is further illustrated by a report of Albaum & Zahl (70) that, in mice implanted with sarcoma 180, although Amethopterin significantly lowered the DNA content of the tumor, it produced similar effects on the spleen. Data of Barton & Laird (71) supports the present view that the action of the 4-aminofolic acid antagonists is directed chiefly against tissues in which there is rapid cell division; Amethopterin did not inhibit nucleic acid synthesis in rats which were recovering from fasting; but when a partial hepatectomy was performed to induce mitosis, the nucleic acid metabolism in the liver was markedly inhibited by Amethopterin. McGlohon *et al.* (72) found that the inhibition of certain microorganisms by certain folic acid antagonists could be reversed by thiamine and vitamin B<sub>6</sub> which suggested to them that the latter vitamins may be concerned in synthesis of polynucleotides by a route other than one requiring the participation of folic acid coenzymes.

*Other compounds having antifolic acid activity.*—Timmis (73) has briefly reviewed the main classes of antifolic acid compounds prepared to date which are of real or potential value in cancer chemotherapy. In addition to the 4-aminofolic acid antagonists these include 2,4 diamino-6,7 substituted pteridines, 2,4-diamino-5-chlorophenylpyrimidines, 4,6-diamino-1-chlorophenyl-1,2-dihydrotriazines, amino-5-arylazopyrimidines (II), Figure 3, and amino-8-aryl-8-azapurines (III), Figure 3. A number of these latter arylazopyrimidines and purines were synthesized (74), but the most active compound had only one fiftieth the potency of Amethopterin as an inhibitor for growth of *S. faecalis*. Modest *et al.* (75) independently prepared some closely related analogues, an interesting example of which was the case where  $R' = \text{NH}_2$  and  $R'' = \text{SH}$ . These analogues have structural features in common with certain folic acid antagonists, such as the diaminopyrimidines, and certain purine antagonists, such as 6-mercaptapurine and azaguanine;

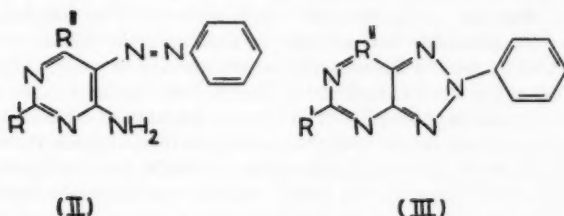


FIG. 3. Examples of 5-arylazopyrimidine (II) and 8-aryl-8-azapurine (III) structures.

moreover, the 2,6-diamino-4-mercapto-5-arylazopyrimidine exhibited both antifolic acid and antipurine activity in appropriate microbiological test systems (75). It will be of interest to learn if this mercaptopyrimidine will show antineoplastic activity. Acheson (76) has extended earlier work of Woolley & Pringle (77) showing that 4-(2-quinoxalinylicarbonylamino) benzoylglutamic acid had some activity as a folic acid antagonist; a number of related quinoxalines were prepared, but none showed pronounced activity. It is apparent that interest still continues in preparing compounds that block folic acid metabolism and, in so doing, be of chemotherapeutic value, but considerably more knowledge of nutritional and biochemical differences between normal and cancer cells is needed to aid a more rational chemical attack in this area.

Earlier reports in the British literature have been confirmed (78, 79) that, in certain instances, a megaloblastic anemia may be induced in patients undergoing treatment with certain anticonvulsant substances structurally related to phenobarbital, and that the anemia responds to treatment with folic acid. Any relationship of these anticonvulsant substances to interference with folic acid metabolism is obscure.

VITAMIN B<sub>12</sub> GROUP

*Chemistry of vitamin B<sub>12</sub> and related factors.*—The complete proceedings of the First European Symposium on Vitamin B<sub>12</sub> and Intrinsic Factor have been published (80), and of particular interest to biochemists will be those chapters on the chemistry and biosynthesis of vitamin B<sub>12</sub> compounds, the biological activity and biochemical mechanism of action of vitamin B<sub>12</sub>, and the intrinsic factor and B<sub>12</sub>-binding factors. The last chapter of this book (80) contains a report of a nomenclature commission, which made the following proposals relative to the nomenclature of vitamin B<sub>12</sub>. It was proposed to call the fundamental ring system of the vitamin, corrin, i.e. the four pyrrol nuclei joined in a macro ring with three bridge carbon atoms and six conjugated double bonds [for a review of the structure of vitamin B<sub>12</sub> see Pfiffner & Bird (81)]. For example, metal derivatives would then be called cobaltocorrin or ferri-corrin, etc. Corphyrin was designated as the generic term for any compound in the series containing the corrin ring system; the term was chosen because it suggests the relationship both to cobalamin and porphyrin. Corphinamide was selected to designate the cobalt-containing porphyrin-like nucleus of B<sub>12</sub> plus the isopropanolamine moiety. Factor B, (vitamin B<sub>12</sub> minus the benzimidazole ribotide) for example, may then be described as cyanocorphinamide. The ribose-3-phosphate ester of corphinamide was termed cobamide. Using this nomenclature, vitamin B<sub>12</sub> could be named 5, 6-dimethyl- $\alpha$ -benzimidazole-cyanocobamide; pseudovitamin B<sub>12</sub>,  $\alpha$ -adenino-cyanocobamide; Factor III, 5-hydroxy- $\alpha$ -benzimidazole-cyanocobamide, etc. Provision was also made for systematically naming the acid degradation products of B<sub>12</sub>. It was proposed to retain the term cyanocobalamin as the official trivial name for vitamin B<sub>12</sub>. It was stated that these recommendations are to be regarded as only provisional, as at present they are awaiting approval of the International Union of Pure and Applied Chemistry Commission and of the International Union of Biochemistry.

The evidence from chemical degradation and x-ray studies leading to the proposed structure for vitamin B<sub>12</sub> was summarized in this volume two years ago (81). Two complete papers have now appeared by the British workers (82, 83) describing in detail the isolation and chemistry of the hexacarboxylic acid fragment of vitamin B<sub>12</sub> which proved to be particularly amenable to x-ray analysis from which the structure of the vitamin was adduced [cf. (81) for references]. Following vigorous alkaline hydrolysis of vitamin B<sub>12</sub>, a mixture of tetra-, penta-, and hexacarboxylic acids were obtained, from which on fractionation by ion exchange the crystalline hexacarboxylic acid was isolated both as the dicyanide and as the monochloride monocyanide. The structure of the latter was deduced by a consideration of the x-ray data, the known chemical properties of the acid and certain analogies in structure to the natural porphyrin, uroporphyrin. The second paper (83) discusses some of the properties of the product of reaction of vitamin B<sub>12</sub> with the halogenating agent, chloramine-T; arguments are presented that the preferred structure for vitamin B<sub>12</sub> is one containing a system of six conjugated double



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Friedrich & Bernhauer have continued their studies on the chemistry of certain analogues of vitamin B<sub>12</sub>. Conditions were described for the mild hydrolysis of certain members of the vitamin B<sub>12</sub> group with ceric hydroxide to give Factor B, nucleoside, and phosphoric acid. By this procedure these workers isolated in crystalline form the nucleoside of pseudovitamin B<sub>12</sub> and Factor A, and concluded that these nucleosides were 7-(D-ribo-furanosyl)-adenine (86) and 2-methyl-7-(D-ribofuranosyl)-adenine (87) respectively. Ultraviolet spectral data led the authors to the conclusion that the pentose was attached to the N-7 of the purine in contrast to the customary linkage to the N-9 position in the purine nucleosides. Other papers from Bernhauer's laboratory described the spectrophotometric and electrophoretic behavior of Factor III (88), and the synthesis, chemical and microbiological properties of a series of phenol ethers of Factor III (89). Friedrich & Bernhauer have isolated another vitamin B<sub>12</sub>-like factor from sewage in which the base of the nucleoside was identified as 2-methylmercaptoadenine (90). This would appear to be the first report of a naturally occurring mercaptopurine, although Ford *et al.* had previously observed that a B<sub>12</sub>-like factor was formed following incubation of 2 mercaptoadenine and Factor B with *E. coli* cells (91). Guanine has been identified most recently as the nucleotide base in a vitamin B<sub>12</sub>-like compound produced during growth of a nocardia strain (92). Yet another B<sub>12</sub>-like factor was isolated from sewage by Dellweg & Bernhauer (93); degradative studies yielded only Factor B, ribose, and phosphoric acid. The factor was accordingly named aetiocabalamin-phosphoribose. It was postulated that in it ribose phosphate is linked via phosphate directly to the cobalamin nucleus (Factor B); the possibility that this factor might have arisen as an artifact during the isolation procedure was considered but not rigidly excluded. Seaman & Sanders (94) have described the properties of a vitamin B<sub>12</sub>-like factor in the haemoflagellate *Crithidia fasciculata*. The factor had a microbiological "spectrum" similar to that of pseudovitamin B<sub>12</sub> but, based on its lability to acid and alkali, the authors suggest that the material may not contain Factor B.

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*Biological properties of factors related to vitamin B<sub>12</sub>; biosynthesis.*—A paper by Coates and Kon (80a), contains a summary of the biological activity of the known available derivatives (1956) of vitamin B<sub>12</sub> for *E. coli* 113-3, *Lactobacillus leichmannii*, *Euglena gracilis*, *Ochromonas malhamensis*, the chick and in pernicious anemia in man. Of the naturally occurring analogues, only Factor III has activity for *O. malhamensis*, the chick and in man; whereas the B<sub>12</sub> factors which have a purine in the nucleotide are inactive for these species but possess varying microbiological activity. A complete

account by the Reading group (95) of the activity for chicks of a number of natural or unnatural analogues of vitamin B<sub>12</sub> has since been published. In a similar study Fox, Briggs & Ortiz (96) are in agreement that Factor III has vitamin B<sub>12</sub> activity (10 to 30 per cent as active) for the chick, and that Factor B is inactive; aceto-, chloro-, nitro-, or sulfatocobalamin were equally as active as cyanocobalamin for chick growth. Coates *et al.* (95) noted that, when Factor B and pseudovitamin B<sub>12</sub> were given at sufficiently high level orally, these factors had some effect antagonistic to vitamin B<sub>12</sub>. Detailed studies of the nutrition of a number of cobalamin-requiring soil bacteria, including the organism referred to in the literature as "Lochead 38" have appeared (97, 98). With only one exception the spectrum of microbiological activity of the B<sub>12</sub> analogues available for study paralleled *O. malhamensis* and higher animals. Ford & Hutner (97) point out, therefore, the potential usefulness of these bacteria to workers in this field because the organisms grow rapidly and can be cultivated in agar media which permit pad plate assays or bioautography, these techniques not being possible with euglena or ochromonas cultures.

A preliminary account of the utilization of  $\delta$ -aminolevulinic acid, a known porphyrin precursor, for the synthesis of vitamin B<sub>12</sub> has appeared by Shemin *et al.* (99). The vitamin was produced microbiologically by growing an unnamed microorganism in a suitable medium containing  $\delta$ -aminolevulinic acid-1,4-C<sup>14</sup>. Nonradioactive B<sub>12</sub> was added to the culture filtrate as carrier to aid in the isolation of the B<sub>12</sub> produced biosynthetically. The radioactivity of the B<sub>12</sub> isolated, in relation to the radioactivity of initial substrate, permitted the conclusion that the porphyrinlike structure of vitamin B<sub>12</sub> was synthesized from  $\delta$ -aminolevulinic acid, and that the mechanism of the synthesis of the ring system in the vitamin was similar to that of the porphyrins. Krasna, Rosenblum & Sprinson (100) have established that the 1-amino-2-propanol moiety of vitamin B<sub>12</sub> arises from the decarboxylation of threonine. *Streptomyces griseus* was grown in the presence of L-threonine N<sup>15</sup>; cyanocobalamin was isolated from the culture filtrate, converted to chlorocobalamin, and the various nitrogen constituents were separated by appropriate degradative procedures. The aminopropanol had a high content of N<sup>15</sup>.

The earlier postulate of Woolley (101) that 1,2-dimethyl-4,5-diamino benzene (DMDAB) may be a precursor of dimethylbenzimidazole in vitamin B<sub>12</sub> receives support from observations of Nakamura (102). *Endamoeba histolytica*, cultured in bacteria-free media, did not require vitamin B<sub>12</sub>, but DMDAB was found to be markedly stimulatory for growth and multiplication. 1,2-Dibromo-4,5-diaminobenzene, an analogue of DMDAB, was inhibitory for growth, but the inhibition was reversed by DMDAB or vitamin B<sub>12</sub>. Curiously enough, 5,6-dimethylbenzimidazole was inhibitory for growth and this effect was partially counteracted by DMDAB or vitamin B<sub>12</sub>. Southcott & Tarr (103) have taken advantage of the fact that certain marine forms of life are usually relatively good sources of the cobalamines and have

studied the biosynthesis of vitamin B<sub>12</sub> by bacillus and micrococcus cultures isolated from fresh clams. These organisms synthesized vitamin B<sub>12</sub> and other cobalamines as determined by paper chromatography and autobio-graphic techniques. Synthesis was stimulated by Factor B and by certain purines or benzimidazoles, particularly 6-nitrobenzimidazole.

*Metabolism and nutritional studies.*—It has been clear from early nutritional experiments that vitamin B<sub>12</sub> is involved in protein metabolism. Yet attempts to obtain supporting evidence at the enzyme level have not been forthcoming, the one notable exception being the evidence from Woods' laboratory for a stimulation of methionine biosynthesis by vitamin B<sub>12</sub> in a cell-free enzyme system of *E. coli* (104). Recent experiments by Wagle, Mehta & Johnson (105) strongly imply that vitamin B<sub>12</sub> is a cofactor for the incorporation of amino acids into protein. The incorporation of C<sup>14</sup>H<sub>3</sub>-methionine or 2C<sup>14</sup>-alanine into protein by a microsome preparation from rat liver and spleen was studied. The complete system employed was essentially that of Keller & Zamecnik (106). There was significantly less amino acid incorporated into protein in those instances where the microsome preparation was derived from tissues of vitamin B<sub>12</sub>-deficient rats; moreover, the *in vitro* addition of vitamin B<sub>12</sub> to the latter microsome preparation markedly enhanced incorporation. Experiments in whole animals further supported a role for the vitamin in protein biosynthesis. When Co<sup>60</sup>-vitamin B<sub>12</sub> was injected into a rat, a study of the distribution of the vitamin in the subcellular fraction of liver showed the vitamin to be concentrated predominantly in the microsomes and supernatant; i.e., in those fractions associated with protein biosynthesis. In other experiments Wagle & Johnson (107) injected serine-3-C<sup>14</sup> into normal or vitamin B<sub>12</sub>-deficient rats and pigs; subsequent analysis showed that the amino acid was present in significantly smaller quantity in the proteins of the liver from the deficient animals and in the serine derived from these proteins. These effects were thought to be specific for vitamin B<sub>12</sub> deficiency, for other experiments (108) had previously demonstrated that, in a severe vitamin A deficiency in the rat, there was no loss in ability to incorporate acetate into protein. Mulgaonkar & Sreenivasan (109) noted a significant reduction in serum proteins (albumin,  $\alpha_1$ -globulin, and  $\gamma$ -globulin) in rats deficient in both folic acid and vitamin B<sub>12</sub>; although conclusions are somewhat uncertain because of the double dietary deficiency, these results may mirror an impairment of protein synthesis in the absence of B<sub>12</sub>.

The early observations that the requirement of certain lactic acid bacteria for vitamin B<sub>12</sub> could be obviated at least in part by various deoxyribosides was taken to imply an involvement of vitamin B<sub>12</sub> in the synthesis of nucleosides. Conflicting reports have recently appeared concerning the possible role of vitamin B<sub>12</sub> in nucleic acid synthesis. In the experiment of Wagle & Johnson (107) described above, the radioactivity of the RNA of the liver from tissues of vitamin B<sub>12</sub>-deficient rats receiving serine-3-C<sup>14</sup> was no different from that of non-deficient controls. Similar results were ob-

tained following injection of other 1-carbon precursors into deficient animals (110). O'Dell & Bruemmer (111) studied the effect of a vitamin B<sub>12</sub> deficiency on nucleic acid and phospholipid metabolism in the newborn rat by measuring the incorporation of P<sup>32</sup> into the brain and liver. The deficiency had no effect on the incorporation of P<sup>32</sup> into phospholipide, but markedly decreased its incorporation into both RNA and DNA. These results were at variance with a similar study by Venkataraman & Barnum (112) who could find no direct effect of a B<sub>12</sub> deficiency on the incorporation of P<sup>32</sup> into the RNA of intestine or spleen, or into liver DNA in rats; moreover, B<sub>12</sub> appeared to have no effect on the amount of DNA or RNA in these tissues. Wong & Schweigert (113) have considered the possibility that vitamin B<sub>12</sub> might be involved in the synthesis of deoxyribose via condensation of glyceraldehyde-3-phosphate and acetaldehyde. Liver homogenates from normal or B<sub>12</sub>-deficient rats were used as source of deoxyriboaldolase enzyme; some evidence was obtained for decreased deoxyribose synthesis in the homogenates from deficient animals. The authors recognize that the enzyme system is crude, but the observations should certainly prompt further study in this area.

Johnson *et al.* (114) have obtained evidence from nutritional experiments with *O. malhamensis* that vitamin B<sub>12</sub> is concerned in methyl group synthesis. This protozoan requires B<sub>12</sub> for growth and is stimulated by methionine. Under appropriate cultural conditions the effects of methionine were duplicated by certain methyl group precursors, principally glycine or serine, but only in the presence of ample vitamin B<sub>12</sub>. Cells grown without methionine but with  $\alpha$ -C<sup>14</sup>-glycine and vitamin B<sub>12</sub> contained C<sup>14</sup>-methionine as show by radioautography. Similar results were obtained following incubation of  $\alpha$ -C<sup>14</sup>-glycine with cell-free extracts of *O. malhamensis* grown with adequate vitamin B<sub>12</sub>. Further microbiological evidence for a metabolic relationship between glycine and vitamin B<sub>12</sub> was found by Alimchandani & Sreenivasan (115). These workers studied the inhibition of *E. coli* Macleod strain with sulfadiazine; in addition to the known noncompetitive antagonists of sulfadiazine (methionine, xanthine, serine, thymine, and valine), it was observed that glycine or vitamin B<sub>12</sub> would further reduce the toxicity of the antimetabolite. The effect is presumably not related to methionine synthesis since the latter was in the medium.

Several papers have appeared concerning vitamin B<sub>12</sub> and certain aspects of fat metabolism. Fox, Ortiz & Briggs (116) have reported that, when undepleted chicks were fed a corn-soybean meal diet supplemented with 20 per cent lard oil for four weeks, the B<sub>12</sub> requirement was increased some ten- to twentyfold. The effect did not appear to be due to interference with the absorption of dietary B<sub>12</sub> or its subsequent storage in the liver. Further work (117) showed that the exaggerated B<sub>12</sub> requirement under these conditions could be lowered to a small extent by choline but completely replaced by 0.15 per cent methionine in the diet; hence it appears that the primary effect of the high fat was to increase the requirement for methionine. In the B<sub>12</sub>-deficient chick the coenzyme-A (118) or pantothenate (119) level in the

liver is elevated; the converse of this relationship has now been demonstrated (120) in that, in pantothenic acid deficiency in the chick, the B<sub>12</sub> content of the liver was twice as great as that of supplemented controls. The metabolic significance of these relationships remains to be determined. The histologic characteristics of the thyroid gland accompanying a vitamin B<sub>12</sub> deficiency in the chick embryo was studied by Ferguson and co-workers (121, 122). Although the gland was larger in deficient embryos than in controls, its rate of development was retarded and there was a reduced ability to concentrate I<sup>131</sup> when compared with thyroid from normal embryos. Vitamin B<sub>12</sub> deficiency in man was discussed briefly by Girdwood (123).

*Assay of vitamin B<sub>12</sub>, clinical aspects.*—Several papers have appeared bearing on various aspects of microbiological assays for vitamin B<sub>12</sub>. Hutner, Bach & Ross (124) found that alterations of the carbohydrate and amino acid content of the basal medium for culturing *E. gracilis* improved the use of this organism for measuring the B<sub>12</sub> content of serum and urine; they recommend the use of a fast growing Z strain of euglena. Additional modifications of this method for assay of B<sub>12</sub> in serum, including use of a more dilute inoculum, digestion of serum samples with papain, and stabilization of released vitamin by heating with metabisulphite, were suggested by Shenoy *et al.* (125). Rogers & Campbell (126) studied various vitamin interrelationships for *L. leichmannii* 313. When folic acid was present at a suboptimal level in the medium, the amount of B<sub>12</sub> normally required for maximum growth exerted a markedly inhibitory effect on growth of the organism. These microbiological findings may have their counterpart in the observations of Harris (127) who reported that certain clinical manifestations of folic acid deficiency were aggravated by small daily doses of vitamin B<sub>12</sub>. A discussion of some of the factors that may increase the precision and reliability of microbiological assays was given in a paper on the calculation of microbial assays by Bliss (128); numerical examples were derived from typical data from vitamin B<sub>12</sub> assays. Full details of a radioisotope assay method for the cobalamins were published (129) together with a collaborative study report which indicated that the method was satisfactory for crude samples known to contain red pigment impurities that may have microbiological activity. The method was based on that of Bacher, Boley & Shonk (130) and consists of the addition and equilibration of a known amount of Co<sup>60</sup>-B<sub>12</sub> to the test sample, conversion of cobalamins to cyanocobalamin, separation of cyanocobalamin from interfering substances by solvent extraction and ion exchange, and measurement of the amounts of cyanocobalamin and remaining tracer.

Improved microbiological assays for vitamin B<sub>12</sub> and tracer studies with Co<sup>60</sup>- and Co<sup>60</sup>-B<sub>12</sub> are being used more widely to obtain data of nutritional and clinical interest. Studies have been made, only a few of which can be mentioned here, on the level of vitamin B<sub>12</sub> in various tissues or excretions from normal individuals (80b, 80c, 131, 132, 133), in mothers (134, 135) and infants (80c, 134) and in patients with various blood dyscrasias including pernicious anemia (80b, 80c, 140), nutritional macrocytic anemia (80b, 136,



137), leukemia and malignant lymphomas (138), cirrheses (132), in rats following carbon tetrachloride injury (139), etc. Mollin & Ross (80b) have summarized the data of a number of prominent clinical investigators on the B<sub>12</sub> content of serum from normal and pernicious anemia patients. Although there was considerable variation, the data clearly showed that the level in the anemic individuals was much lower than in normal subjects; e.g., data from their own laboratory indicated serum-B<sub>12</sub> concentrations ranging from 100 to 900  $\mu\text{g.}/\text{ml.}$  in 223 normal individuals and ranged from 16 to 110  $\mu\text{g.}/\text{ml.}$  in 254 untreated patients with pernicious anemia. Gaffney and co-workers (131) determined the vitamin B<sub>12</sub> serum levels of 528 apparently healthy individuals varying in age from 12 to 94; their results indicated a regression of B<sub>12</sub> level with age. The distribution of vitamin B<sub>12</sub> in various tissues obtained posthumously, including liver, kidney, heart, spleen, and brain in adults, infants, and in pernicious anemia patients, was determined by Ross & Mollin (80c). In 13 adult controls the content of B<sub>12</sub> in the whole liver ranged from 486 to 4330  $\mu\text{g.}$  which contrasted with 10  $\mu\text{g.}$  B<sub>12</sub> found in the liver of a patient who had died from untreated pernicious anemia. In an extensive study Swendseid *et al.* (132) determined the average vitamin B<sub>12</sub> content of liver tissue (autopsy samples) of 132 males to be 0.7  $\mu\text{g.}/\text{gm.}$  of wet tissue. There was no significant difference in the B<sub>12</sub> stores between the age groups. On the basis of certain assumptions, the authors calculated that there was sufficient B<sub>12</sub> in liver to meet the requirement of a pernicious anemia patient for three years, which is in good agreement with clinical findings that there is a considerable storage of B<sub>12</sub> in terms of nutritional requirement. Boger and co-workers (134) have published extensive data with which Baker *et al.* (135) are in agreement, that the vitamin B<sub>12</sub> level in serum is lowered during pregnancy. The latter workers (135) also found that the serum folic acid level in pregnancy was two to eight times higher than the reported normal range. A relationship between B<sub>12</sub> and folic acid has been observed in a series of patients with nutritional macrocytic anemia by Narayanan, Shenoy, & Ramasarma (136, 137). The intramuscular injection of two 100-microgram doses of vitamin B<sub>12</sub> on consecutive days in these patients produced a significant rise in the serum folic acid; when the patients were subsequently given two daily intramuscular injections of 25 mg. folic acid, there was a gradual rise in the B<sub>12</sub> level in the serum, suggesting that large doses of one of these vitamins tends to mobilize the other from the body stores. These results may be contrasted with a report last year of Lear & Castle (141) suggesting that the level of B<sub>12</sub> in the serum of pernicious anemia patients may be reduced by the administration of folic acid although the different experimental conditions employed in these studies (137, 141) makes comparisons difficult. This latter paper, together with other pertinent observations bearing on clinical and biochemical relationships between vitamin B<sub>12</sub> and folic acid, were ably discussed by Welch (142).

Booth & Mollin (80d) administered Co<sup>60</sup>-B<sub>12</sub> orally to appropriate test patients and then measured the radioactivity of the plasma as an indication



of ability to absorb vitamin B<sub>12</sub>. In control patients absorption was at a peak in 8 to 12 hours and was markedly higher than that of individuals with pernicious anemia; Goldberg *et al.* (143) concur with these findings. It was suggested (80d, 143) that this method for studying possible impairment of B<sub>12</sub> absorption has certain advantages over other methods such as hepatic radioactivity (144) or urinary excretion (145) methods.

Studies are continuing on the nature and extent of binding of vitamin B<sub>12</sub> by the serum proteins; such work is important to an understanding of the transport mechanism of the vitamin, and may lead to clinical applications (146). Miller (147) has presented a modification of the microbiological procedure of Rosenthal & Sarett (148) for the determination of "bound" B<sub>12</sub> in serum based on the observation that B<sub>12</sub>, in the free but not in the bound state, is adsorbed by charcoal. It was estimated that 80 to 85 per cent of the vitamin B<sub>12</sub> activity in serum of normal individuals is bound, which is in general agreement with results of other workers. Bertcher & Meyer (149) incubated samples of serum with Co<sup>60</sup>-B<sub>12</sub> in a dialysis bag and then determined the radioactivity remaining after dialysis; a greater binding capacity was found by this method than has been indicated by microbiological procedures. Experiments on the *in vitro* binding of vitamin B<sub>12</sub> by kidney homogenates (150, 151) and rat liver slices (151, 152, 153) have been briefly described. Miller & Hunter (151), Latner & Raine (152), and Herbert & London (153) have all reported that radioactive B<sub>12</sub> is taken up by rat liver slices under appropriate conditions; the uptake appeared to be enhanced by intrinsic factor preparations. The latter observation is of considerable importance if this proves to be specific for pure intrinsic factor, for these findings suggest (151) a possible rapid assay for evaluating the potency of intrinsic factor preparations; and moreover, these reports would appear to establish an intrinsic factor-B<sub>12</sub> relationship in tissues other than intestinal mucosa. Herbert & London (153) used an intrinsic factor concentrate with a clinical potency of 1 mg. (daily oral dose); with this preparation an enhancement of up to tenfold of Co<sup>60</sup>-B<sub>12</sub> uptake by rat liver slices was found and was not duplicated by certain mucoproteins.

The most comprehensive summary of the present status of purification of intrinsic factor is to be found in a series of papers in the aforementioned symposium (80). The papers of Wijmenga (80e), and Williams & Ellenbogen (80f) indicate the extensive efforts aimed at purification. These latter workers (80f) successfully extended the technique of Sober and Peterson (154) of chromatographing proteins on a DEAE-cellulose ion exchange column to the problem of intrinsic factor purification, and by this procedure have obtained a highly purified preparation which, on the basis of activity in the urinary excretion test (155), was estimated to be active in pernicious anemia patients at a daily oral dose of 1 mg. Recent work of Holdsworth (156) and Robbins & Shields (157) also describe the preparation of purified intrinsic factor by cellulose ion exchange columns; the concentrate obtained by the latter workers, however, was active only at a daily oral dosage of 9 mg. As an aid

in the isolation of intrinsic factor Holdsworth (156) added  $\text{Co}^{60}$ -vitamin  $\text{B}_{12}$  as a marker at the beginning of the fractionation procedure, and followed the radioactivity during the subsequent isolation procedure. A fraction was obtained with clinical activity, one mg. of which was capable of combining with  $15\gamma$  vitamin  $\text{B}_{12}$  to form a stable complex. Until pure material is obtained, however, the relationship between intrinsic factor and binding of vitamin  $\text{B}_{12}$  or absorption cannot be clearly defined in chemical or physiological terms. Nieweg, Shen & Castle (158) present evidence from experiments with gastrectomized rats that suggests that the mechanism of action of intrinsic factor may involve a relationship between intrinsic factor and the surface of the intestinal mucosa. Citrin, DeRosa & Halsted (159) administered  $\text{Co}^{60}$ - $\text{B}_{12}$  to normal and pernicious anemia patients by intestinal intubation to various levels of the intestinal tract to determine the locus of absorption of vitamin  $\text{B}_{12}$ . The results indicated that the jejunum and ileum as well as the stomach and duodenum are capable of absorption of vitamin  $\text{B}_{12}$ , but not the large bowel, and that the absorption from these loci in pernicious anemia patients requires the presence of intrinsic factor.

#### CHOLINE

This portion of the review will be restricted to a consideration of some of the nutritional aspects of choline, particularly as they relate to folic acid and vitamin  $\text{B}_{12}$ . Jukes (160) has recently summarized present views of the biochemistry of methyl group transfer and methyl group synthesis which are pertinent to this discussion. As may be seen in the accompanying diagram (Fig. 4), methyl groups for the formation of methionine are supplied either in the preformed state as choline or betaine in the diet, or by *de novo* synthesis in the form of the postulated hydroxymethyltetrahydrofolic acid. Homocysteine can accept methyl groups from either of these separate sources; hence, there are two processes for the methylation of homocysteine. The first is by transmethylation with an intact methyl group by means of the enzyme methyltransferase. The second way in which homocysteine receives a methyl group is by reduction of the single carbon unit of the folic acid coenzyme. Vitamin  $\text{B}_{12}$  appears to be involved in this second procedure. This dual possibility explains why animals that are deficient in methionine may respond either to folic acid plus vitamin  $\text{B}_{12}$ , or to choline (or betaine).

Yet a third procedure is involved in the further pathway of the methyl group of methionine. In this step, S-adenosylmethionine transfers its methyl group to "methyl receptors" such as glycocyamine, dimethylaminoethanol and nicotinamide.

Two of the methyl groups of choline are apparently derived from the folic acid coenzyme which methylates aminoethanol to form diaminoethanol as suggested by experiments of Stekol *et al.* (161). The third methyl group of choline is derived from S-adenosylmethionine as shown in the diagram.

Although the route for the biosynthesis of choline from aminoethanol and methionine, via the transformations shown in Figure 4 is suggested from

numerous experiments with intact animals, supporting data are now beginning to appear from appropriate *in vitro* experiments. Pilgeram, Hamilton & Greenberg (162) studied the *in vitro* synthesis of choline from ethanolamine-1,2- $C^{14}$  by rat liver slices; the system allowed the further conversion of

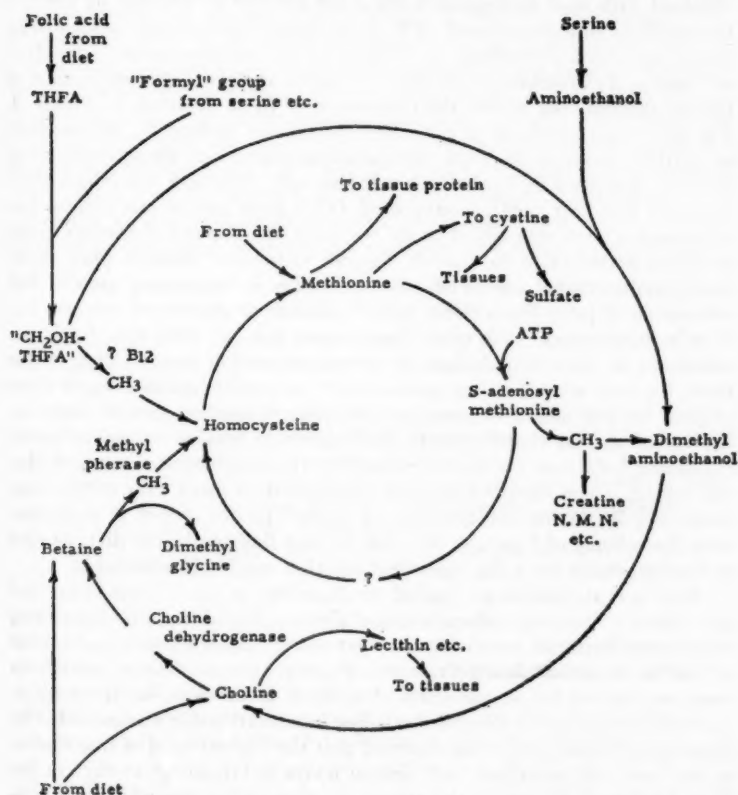


FIG. 4. Aspects of methyl group biogenesis and transfer, after Jukes (160).

choline to phosphatidylcholine (lecithin), but the study was directed toward the factors concerned in choline biosynthesis. In the absence of methionine, ethanolamine was not appreciably converted to phosphatidyl choline (0.04 per cent conversion in contrast to 2.14 per cent in *in vivo* experiments); but, following the addition of methionine, there was a nineteenfold stimulation in choline synthesis. Homocysteine was 52 per cent and betaine 34.5 per cent as effective as methionine in enhancing phosphatidylcholine formation from

ethanolamine, and homocysteine and betaine had a synergistic effect when added together. The effect of homocysteine could not be duplicated by cysteine, and therefore its effect was not attributable to the SH group alone. Phosphatidylcholine synthesis from ethanolamine and methionine was not obtained with liver homogenates nor could activity be restored by the further addition of folic acid and ATP, which, from Figure 4, can be seen to be logical cofactors. Doubtless, purification of the enzyme systems involved will lead to a clarification of the more intimate aspects of the mechanism of choline biosynthesis within the framework of those depicted in Figure 4. The enzymatic synthesis of cytidine diphosphate choline an intermediate in lecithin biosynthesis, from cytidinetriphosphate and phosphorylcholine has been described by Borkenhagen & Kennedy (163) and was reviewed by Kennedy last year (164). Young *et al.* (165) have carried out appropriate experiments with rats aimed at determining the amount of methyl group synthesis attributable to vitamin B<sub>12</sub> and to betaine. Betaine plus monomethylaminoethanol was as effective as choline in supporting growth and prevention of fatty livers either in the presence or absence of vitamin B<sub>12</sub>. This is in agreement with other observations [e.g., cf. (39)] that B<sub>12</sub> is not concerned in transmethylation. In experiments with rats on diets where methyl groups were limiting, such as with monomethylaminoethanol alone or plus betaine added in marginal amounts, a marked growth response, equivalent to that contributed by 0.077 per cent betaine, was obtained to B<sub>12</sub> although the liver fat was not reduced to the same extent as when choline was added. These experiments were interpreted to mean that under these conditions B<sub>12</sub> favors the synthesis of methyl groups, which is consistent with the scheme of Figure 4, for with betaine limiting in the diet, *de novo* methyl synthesis via a B<sub>12</sub>-dependent reaction can be demonstrated.

Benton *et al.* (166) have studied the deposition of fat in livers of rats fed fatty diets containing various levels of choline. Rats fed a diet containing 30 per cent butterfat required somewhat more choline to reduce the level of liver fat to normal than did rats on a 30 per cent corn oil diet; these effects were not altered by supplements of cystine, methionine, or tryptophan. Evidence for a possible vitamin A-choline interrelationship was provided by Drasnin & Krause (167) who observed that the highest level of free choline in rat liver was associated with normal levels of vitamin A storage in the liver. Choline deficiency in the guinea pig was further described in experiments by Young & Lucas (168). Four- to six-day-old guinea pigs on a choline-deficient diet grew slowly and died within three to four weeks; an interesting finding was that the liver at time of death showed only traces of stainable fat. Earlier work of Reid (169) was extended and showed that the guinea pig, like the chick, is able to utilize monomethylaminoethanol plus betaine (168) or methionine (169) in lieu of choline, but cannot utilize ethanolamine. Hodgson *et al.* (170) reported that the insect *Phormia regina* requires choline for growth; carnitine or dimethylaminoethanol, but not betaine, could substitute for choline. The activity of carnitine in this instance contrasts with a

report (171) that, in the rat on a choline-deficient diet, carnitine was unable to prevent fatty infiltration into the liver. Ethionine, a methionine antagonist, has been effectively used in nutritional experiments as a choline antagonist (172, 173). Salmon & Copeland (173) found that the addition of 0.05 to 0.2 per cent ethionine to diets containing 0.2 per cent or less choline markedly inhibited growth of rats and produced lesions typical of choline deficiency. These effects of ethionine could be counteracted by methionine or choline, and furthermore, the action of the latter metabolites was synergized by vitamin B<sub>12</sub>. It would appear that the action of ethionine is particularly directed against the ability of methionine to function as a methyl donor for choline synthesis. Strength has briefly reported (174) that betaine aldehyde is a product of the oxidation of choline by rat liver mitochondria. The aldehyde was isolated from the reaction mixture as the reineckate and subsequently converted to the 2,4-dinitrophenyl-hydrazone derivative which permitted its isolation and identification and also suggested a method for its assay in tissues.

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## WATER-SOLUBLE VITAMINS, PART II<sup>1,2,3</sup> (ASCORBIC ACID, BIOTIN, NICOTINAMIDE, VITAMIN B<sub>6</sub>)

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The major emphasis in biochemical research during the past year has been in the fields of lipid and nucleic acid metabolism, with decreasing levels of effort in studies on the metabolism of the B vitamins and their derivatives. While this trend away from biochemical and nutritional aspects of B vitamins and amino acids has been evident for several years, it is of interest that within the group of water-soluble vitamins considered in this chapter, the amount of research on the metabolism of ascorbic acid has increased markedly, while relatively little attention was given to biotin, as in preceding years. The two research areas in which greatest progress has been made appear to be on the mechanism of biosynthesis of ascorbic acid and on the role of tryptophan metabolites as precursors of nicotinic acid.

### ASCORBIC ACID

*Chemistry and methods.*—Barr & King studied the oxidation of aqueous ascorbic acid solutions by Co<sup>60</sup>  $\gamma$ -rays and reported no indication of chain oxidation with molecular oxygen under these conditions (1). They suggest that the antioxidant protective action of ascorbic acid, as well as of thiourea, tocopherol and hydroquinone, is probably due to the fact that these compounds are easily oxidized to stable, one electron oxidation products without initiating chain reactions.

Kelley & Watts reported that a Cu-ascorbic acid complex appeared to be the causative agent for the prooxidant effect of ascorbic acid in aqueous fat systems (2). Compounds which exhibited effective antioxidant activity in the presence of ascorbic acid were shown to form stable copper chelates. It was proposed that the formation of free radicals by dehydrogenation from the activation of the methylene groups by two adjacent double bonds in polyethenoic fatty acids may be followed by addition of oxygen to the methylene radical, and that this is then followed by further dehydrogenation and the initiation of a continuous cycle. Ascorbic acid may be involved in the dehydrogenation reaction, with Cu<sup>++</sup> catalyzing the oxidation of the ascorbic acid; the formation of stable copper chelates would then inhibit this oxidative reaction scheme with net antioxidant effects.

<sup>1</sup> Journal Paper No. 158, American Meat Institute Foundation.

<sup>2</sup> The survey of research literature pertaining to this review was completed in October, 1957.

<sup>3</sup> The following abbreviations are used in this chapter: ACTH for adrenocorticotrophic hormone; and DPN for diphosphopyridine nucleotide.

Peel & Loughman (3) suggested further modifications of the conditions for reduction of phosphomolybdate by ascorbic acid in the determination of inorganic phosphate. Miyamoto *et al.* (4) investigated the destruction of ascorbic acid by heat treatment in the presence of HCl and found it to be complete. These authors concluded that the indophenol-reducing substance formed by the heat treatment of wheat germ in HCl is not ascorbic acid. Oesterling reported that ascorbic acid ( $10^{-3}$  M) accelerates the rate of destruction of epinephrine and norepinephrine from acetate-buffered solutions (pH 4-5), and concluded that the  $H_2O_2$  formed by the Cu-catalyzed atmospheric oxidation of ascorbic acid was largely responsible for this effect, since treatment with equimolar amounts of  $H_2O_2$  resulted in equivalent destruction (5).

*Ascorbic acid in human nutrition and metabolism.*—Huisman & Jonxis reported a study of tyrosine and phenylalanine metabolism in scorbutic infants (6). It was found that the urinary excretion of tyrosine, *p*-hydroxyphenyllactic acid and other tyrosine metabolites was markedly increased following tyrosine ingestion in excess. Similar results were observed following the ingestion of large amounts of phenylalanine, along with the urinary excretion of three to five times the amount of phenylalanine observed in normal infants. These authors suggest that renal failure to reabsorb tyrosine may be a specific factor in scurvy.

Namyslowski reported significant reductions in plasma ascorbic acid levels in athletes following intense and moderate physical exertion (7). Morse *et al.* found that serum ascorbic acid levels rose significantly in both younger and older women placed on regimens of increasing ascorbic acid content. White blood cell ascorbic acid levels also increased, but the rise was proportionately less than in the serum (8).

Alsop & Burrill (9) reported that the maximum urinary excretion of ascorbic acid in older women did not exceed 70 per cent and was usually between 50 to 60 per cent of the daily test dose of 10 mg./kg. The maximum excretion values were observed between the fourth and seventh day of treatment, after which the urinary excretion of ascorbic acid decreased continuously in spite of continued administration of the vitamin. Burlina (10) studied 20 aged healthy men and women and found very low blood ascorbic acid levels (0.13 mg./100 ml.) on daily intakes of 30 to 35 mg. of the vitamin. Urinary excretion ranged from about two-thirds to nearly all of the ascorbic acid ingested. This author concluded that hypovitaminosis C may be related to the physiological processes of ageing. It would appear, however, that the patients studied may have had suboptimal dietary intakes of the vitamin.

Schaus analyzed human pituitary glands, cerebral cortex, myocardium and pectoral muscle for ascorbic acid (11). He found significant correlations of the decreasing ascorbic acid contents of pituitary and cerebral cortex with increasing age, and reported that no significant decreases appeared to occur in heart muscle after the second decade of life, nor in skeletal muscle following the fourth decade. Although these findings lend support to Burlina's

conclusions (10), information on the nutritional history and ascorbic acid intake of the patients prior to death was not available.

Ramsay *et al.* reported that on adequate dietary intakes of ascorbic acid, chronic mental patients of all ages had low plasma ascorbic acid values, tissue unsaturation and increased capillary fragility. Ten weeks' treatment with 1 gm. ascorbic acid daily gave significant increases in the blood levels and tissue uptake of ascorbic acid, but was not correlated with the reductions in capillary fragility which were observed (12).

Eales (13) reported that in 14 patients with scurvy, glomerular filtration rate and effective renal plasma flow were somewhat reduced, and reverted toward normal on treatment with ascorbic acid. This author suggests that these results may be ascribed to the remission of the associated scorbutic anemia, as well as to increased protein consumption on the hospital diet.

In a study of 10 normal patients and 12 patients with diagnosed heart disease, Mel (14) found that administration of 10 gm. ascorbic acid produced a 25 per cent reduction in blood potassium levels of the normal patients and was without effect on those with heart disease. He suggested that this represented normal and impaired adrenal function in the two groups, respectively. It would appear to offer further clues to some of the problems currently under active investigation in the area of the relation of nutrient intake and balance to the development of heart disease.

Anderson *et al.* (15) found that in a diet supplemented with 1 gm. of ascorbic acid, serum cholesterol levels in men decreased significantly when safflower oil or selectively hydrogenated safflower oil was substituted isocalorically for carbohydrate, with significantly greater decreases observed for the untreated safflower oil. In the absence of the ascorbic acid supplement, the decreases observed were from one-third to one-half as great, and not significantly different for the two oils tested.

Davey *et al.* (16, 17) reported a comprehensive study of the utilization of ascorbic acid from 24 vegetable foods by human subjects. Using as criteria total blood ascorbic acid, and urinary excretion of ascorbic acid and reduced ascorbic acid, these authors found the ascorbic acid in each of the 24 foods tested was utilized equally as well as synthetic ascorbic acid.

*Ascorbic acid synthesis in plants.*—Mirimanoff (18) reported that ascorbic acid is not synthesized by the chloroplasts in plant cells and indicated that it arises from a hexose precursor. Loewus & Jang (19) found that in both cress seedlings and ripening strawberries the pathway from glucose to ascorbic acid proceeded without cleavage, the aldehydic carbon of D-glucose-1-C<sup>14</sup> forming the carboxyl carbon of L-ascorbic acid. The reaction sequence and enzymatic pathways followed in this conversion of glucose to ascorbic acid in plants are not yet known. These authors (20) also reported that while the ascorbic acid formed from D-glucose-1-C<sup>14</sup> or -2-C<sup>14</sup> possessed 70 per cent of the radioactivity in C-1 or C-2, respectively, significantly greater randomization was observed in the ascorbic acid formed from D-galactose-1-C<sup>14</sup>. They suggest that the initial hexose portion of the oxidative pathway is re-

sponsible for the synthesis of ascorbic acid in plants, and that galactose and glucose each form a portion of the hexose pool, in which glucose is appreciably more stable, however, than galactose.

*Ascorbic acid content of foods.*—In an attempt to improve calcium intakes of humans on essentially vegetable diets, Goto & Honda (21) studied the effect of additions of 0.2 per cent  $\text{CaCO}_3$  to cabbage. Under these conditions, minimal losses of ascorbic acid occurred on cooking, and no off-odor or off-flavor production was encountered, but it was found that the ascorbic acid content decreased very rapidly on standing. Thus, this approach to the problem of calcium fortification of the diet would appear to have potentially serious hazards in terms of the possibility of substantial destruction of the ascorbic acid normally ingested.

There has been a great deal of interest in the content and stability of ascorbic acid in foods, including milk and infant formulas, orange juice, tangerine juice, powdered citrus juices, potatoes, potato granules, sweet potatoes, tomatoes, apricots, cabbage, mangoes, and several foods native to other countries (22 to 34).

*Animal nutrition, synthesis and metabolism.*—Burns & Evans (35) found that in the rat carboxyl-labeled D-glucuronolactone and L-gulonolactone are converted to carboxyl-labelled L-ascorbic acid, while similarly labelled D-glucuronic acid and L-gulonic acid are not, thus indicating the importance of the lactone structure for the biosynthesis of the vitamin. No conversion of the labelled lactones to ascorbic acid could be detected in the guinea pig [Burns *et al.* (36)]. Burns also reported that rats treated with chloretone or barbital could convert D-glucose-1- $\text{C}^{14}$  to urinary L-gulonic acid to an extent of approximately 0.3 per cent, while untreated animals could not. Guinea pigs and rats were found capable of converting preformed D-glucuronolactone to urinary L-gulonic acid to an extent as high as 3 per cent, without treatment with these or other L-ascorbic acid synthesis-stimulating drugs (37).

Using labelled compounds, Dayton showed that no appreciable conversion of 2-keto-L-gulonic acid to L-ascorbic acid occurs in the rat or the guinea pig, and suggested that the most likely intermediate in the conversion of L-gulonolactone to L-ascorbic acid is 2-keto- or 3-keto-L-gulonolactone (38). Chatterjee *et al.* (39) reported that when rat liver homogenate is incubated with D-glucuronolactone, in the presence of KCN ( $5 \times 10^{-2}$  M), from two to three times as much ascorbic acid as was originally present is found. In the absence of KCN, no increase was observed. They conclude that the biosynthesis of ascorbic acid may be catalyzed by a cyanide-activated enzyme system, or that cyanide blocks an alternative pathway for the metabolism of D-glucuronolactone, or both. Goat liver was found to be exceptionally active in this synthesis, while the livers of guinea pig, pigeon and chick were found to be inactive under the conditions employed.

Grollman & Lehninger (40) studied the enzymatic synthesis of L-ascorbic acid from D-glucuronic acid in animal tissues and found that in mammals requiring no dietary ascorbic acid, the liver contains the three enzymes or



enzyme systems needed to effect the conversion. The kidney can convert D-glucuronate to L-gulonate, and L-gulonate to the presumed 3-keto-L-gulonate, but cannot complete the conversion of this intermediate to L-ascorbic acid. In the chicken and pigeon, and in the turtle, only the kidney contains all three enzymes. In man, two species of monkey, and the guinea pig, it was found that the final reaction of the oxidation product of L-gulonate, presumably 3-keto-L-gulonate, does not occur in the liver, although the two preceding reactions do take place. Thus, failure to form ascorbic acid in these species is due to a relative metabolic defect, and the dietary requirement is not due to an unusually rapid rate of catabolism of L-ascorbic acid, as has been suggested previously by other investigators. These studies provide important information on the nature of the enzymatic reaction pathways involved in the biosynthesis of ascorbic acid and on the fundamental reasons for the observed differences in species requirements for preformed ascorbic acid in the diet.

Ganguli *et al.* (41) reported that when pyruvamide-2-C<sup>14</sup> was injected into chloretonized rats, the urinary ascorbic acid, dehydroascorbic acid-2,4-dinitrophenylhydrazone, and glycogen were found to possess 315, 110 and 70 counts/min./mg., respectively, thus suggesting that pyruvate may play an important role as precursor in the biosynthesis of ascorbic acid.

Chan *et al.* (42) reported experiments with guinea pig liver slices *in vitro*, using variously-labelled ascorbic acid. These workers found that 25 per cent of the activity of 1-C<sup>14</sup>-labelled ascorbic acid appeared as CO<sub>2</sub> within one hour, while only negligible amounts arose from the 2,3,4,5,6-labelled compound. In 24-hour urine samples, 95 per cent of the activity of the latter compound was found in the carboxyl group of glycine, compared to 40 per cent for the 1-labelled compound. The 3,4,5,6-labeled ascorbic acid yielded uniformly labeled glycine, indicating that a large proportion of the glycine formed was derived from carbons other than 1 and 2.

In a study of the relation of physiological stress to the reaction kinetics of ascorbic acid catabolism, Salomon (43) found that first-order reaction rates were observed in normal, scorbutic, and diphtheria-intoxicated guinea pigs, and that neither toxic stress nor scurvy affected the biological half life of ascorbic acid in the animal.

In *in vivo* studies with the rat, Thangamani and Sarma (44) found that pantothenic acid is required for the conversion of glucose cycloacetate and of glucuronic acid to ascorbic acid. Lahiri & Banerjee (45) reported that treatment of rats with aminopterin resulted in decreased urinary excretion of ascorbic acid, indicating interference with the biosynthesis of the vitamin in aminopterin-induced pteroylglutamic acid deficiency states. Bhattacharya (46) concluded that ascorbic acid supplementation of the diets of thiamine-deficient rats can maintain normal ascorbic acid blood levels, but is without effect on blood pyruvate levels.

Bodur *et al.* (47) found that the kidney retention and urinary excretion of injected C<sup>14</sup>-oxalic acid in the rat were unaffected by massive doses of

ascorbic acid, and that no endogenous oxalic acid formation was detected in animals so treated. Khalil reported (48) that in hypophysectomized rats, postoperative adrenal weight loss was minimized by intravenous injections of 100 mg. of sodium ascorbate in saline for 7 days. Burns *et al.* (49) found that the administration of barbital produces markedly increased urinary excretion of L-ascorbic acid and D-glucuronic acid in rats and dogs, and of the latter compound in guinea pigs. These authors suggest that barbital and various barbiturates, chlorotone and other drugs may act indirectly on carbohydrate metabolism, with the result that increased amounts of D-glucose are metabolized via D-glucuronolactone.

Straumfjord & West (50) reported that the biosynthesis of ascorbic acid is not impaired in the alloxan-diabetic rat, and concluded that the conversion of glucose to glucose-6-phosphate is not required for this synthesis. Allegretti & Gabric (51) administered ascorbic acid with alloxan or pyromecasonone or both to rats and found reduced red cell counts and increased leukocytes with all treatments. In the guinea pig, they found that ascorbic acid plus alloxan did not produce diabetes, but mortality increased, and borderline anemia may have developed (52).

Tucker *et al.* (53) and Greenberg *et al.* (54) found that in milk-fed anemic rats, the rate of hemoglobin regeneration was consistently higher in animals supplemented with iron plus ascorbic acid and vitamin E than with iron plus either of these or with iron alone. Hemoglobin levels were also better sustained following cessation of iron supplementation in the same animals. Thus, ascorbic acid appears to be clearly implicated in iron absorption and metabolism, in addition to the role it may play in maintaining normal capillary strength and function.

In studies with guinea pigs, Reiff & Free concluded that isoascorbic acid is not antiscorbutic and cannot replace ascorbic acid in the diet of the guinea pig (55). They suggest that it may exert a protective effect on diets containing marginal quantities of ascorbic acid, however, and that it may extend the depletion period in animals which are completely deprived of the vitamin.

Matsko *et al.* (56) found that the weight gains of guinea pigs given ascorbic acid daily, semiweekly, or weekly indicated increased requirements for the vitamin when given at the less frequent intervals. This would be expected in the case of ascorbic acid and other water-soluble vitamins, which cannot be extensively stored within the body. At high daily dose levels, these authors found that weight gains achieved were greater on 20 mg. than on 30 mg. ascorbic acid, indicating that some growth inhibition may occur at very high intake levels of this vitamin.

Chalopin (57) reported that nicoscorbine, a complex of nicotinamide and ascorbic acid, is more effective in maintaining hepatic and adrenal reserves of ascorbic acid than ascorbic acid alone. Nigeon-Dureuil *et al.* (58) reported that nicoscorbine is superior to either nicotinamide or ascorbic acid in curing the hemorrhagic lesions of scurvy, and that it possesses antiscorbutic activity equivalent to that of equimolar amounts of ascorbic acid.

Ratsimamanga & Nigeon-Dureuil (59) reported that in scorbutic guinea pigs, adrenal dysfunction is due to deranged corticosteroid metabolism. Clayton *et al.* reported finding ACTH in the serum of acutely scorbutic guinea pigs, while none was found in the serum of normal controls. Administration of ascorbic acid to the scorbutic animals produced marked reductions in serum ACTH levels (60). Bacchus found that in mild ascorbic acid deficiency, guinea pig adrenal preparations regained the ability to beta-hydroxylate 11-deoxycortisol to cortisol at a normal rate on addition of ascorbic acid *in vitro*, while adrenal preparations from severely deficient animals did not recover this ability (61). This author also reported a progressive decrease in the ability of such preparations to oxidize delta-3-beta-ol steroid compounds to delta-3-ketones with progressive ascorbic acid deficiency. *In vitro* addition of ascorbic acid corrected this metabolic defect (62).

Booker *et al.* (63) reported that massive doses of ascorbic acid produced hypercholesteremia in rats, guinea pigs and humans, and suggested that liver cholesterol release under these conditions may be related to adrenal hormone function. Chronic administration of cholesterol to rabbits and guinea pigs was found to depress the ascorbic acid content of blood cells, plasma, or both, while simultaneous treatment with ascorbic acid and cholesterol minimized this effect. Thus, this work provides further indications that ascorbic acid metabolism and steroid synthesis and metabolism are closely interrelated.

From *in vitro* studies of pig and horse adrenal medulla, Katsura (64) concluded that ascorbic acid functions there to prevent epinephrine oxidation. Oxygen consumption and epinephrine oxidation were more rapid in adrenal medulla homogenates from ascorbic acid-deficient animals than in those prepared from normal and ascorbic acid-treated animals. Karg, however, reported that a deficiency of ascorbic acid had no effect on the epinephrine content of the adrenals in the guinea pig (65). Thus, a homeostatic mechanism may operate here, maintaining normal epinephrine concentrations even when abnormally large destruction of the compound occurs, in ascorbic acid deficiency.

Gould & Woessner (66) found that appreciable formation of hydroxyproline occurs in experimental wounds in guinea pigs placed on scorbutigenic rations at the time of wounding. Depletion for four or seven days previous resulted in considerable impairment and almost complete cessation of hydroxyproline synthesis, respectively. Impaired hydroxyproline formation may thus be one of the first detectable signs of ascorbic acid deficiency. Dinning *et al.* found that a deficiency of ascorbic acid in the diet of the guinea pig resulted in reduced utilization of formate for the synthesis of serine and methionine by liver homogenates (67). Formate incorporation into purines was unaffected by the deficiency, however. The skin and cartilage content of acid-mucopolysaccharide was reported by Bowness to be 15 to 20 per cent lower in scorbutic guinea pigs than in the control animals (68).

Lahiri & Banerjee (69) found that in the scorbutic guinea pig, the inor-

ganic P of blood and liver and hepatic glucose-6-phosphatase activity increase, and decreased liver levels of glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate are observed. Sokolova (70) reported appreciable reductions in the adenosinetriphosphatase activity of heart and skeletal muscle from ascorbic acid-deficient guinea pigs. Barbieri (71) found that liver levels of pyridine nucleotides and coenzyme A were reduced in scorbutic guinea pigs. He also reported that administration of fructose increased the pyridine nucleotide and succinic dehydrogenase levels to normal, and reduced the high liver levels of lactate, alpha-ketoglutarate and pyruvate, while glucose was without effect (72). These studies indicate that ascorbic acid is required for the normal metabolism of carbohydrate and amino acids but further information is required to establish the specific biochemical functions which it fulfills.

Sarkar & Banerjee (73) indicated that the scorbutic monkey is unable to utilize glucose efficiently, as evaluated by the glucose tolerance test. Thus, this work provides further evidence that ascorbic acid is also required for normal carbohydrate metabolism in monkeys. Behki & Nath (74) reported that the condensation product of glucose and acetoacetate, or a hydrolysate of this product, is protective against the diabetes caused by dehydroascorbic acid, and its administration leads to reduced excretion of diketogulonic acid with concomitantly increased excretion of dehydroascorbic acid. Incubation of the glucose-acetoacetate condensate-hydrolysate with dehydroascorbic acid *in vitro* gave ascorbic acid, thus giving a substantial clue to the mechanism of the protective action observed.

Harris *et al.* (75) reported that an attempt to produce ascorbic acid deficiency in the rabbit was unsuccessful, and that this animal is capable of synthesizing adequate amounts of ascorbic acid to meet its requirements. Rousell (76) reported that *Periplaneta americana* L. can synthesize its own ascorbic acid requirements using any of several monosaccharides or sucrose as precursor.

#### BIOTIN

Little attention is currently being given to the role of biotin in metabolism. While many suggestions have been made for its mode of action, no clear-cut biochemical mechanism has as yet been established. Significant contributions have been made recently, particularly in the follow-up of earlier studies in the laboratories of Williams and Lichstein and their associates.

Further evidence for the role of biotin in reactions involving CO<sub>2</sub> has been reported. Lichstein (1) has shown that the amount of bound biotin is significantly correlated with the purity of oxalacetic decarboxylase preparations. Moat *et al.* (2) have indicated that an alteration in purine synthesis by biotin-deficient *Saccharomyces cerevisiae* may involve biotin in the carboxylation of 4-aminoimidazole to form 4-aminoimidazole carboxylic acid, which in the presence of biotin and an amino group donor forms the carboxamide derivative. These conclusions were made from studies with growing or

resting cells of biotin-deficient yeast in which the aromatic amine that accumulated was characterized as 4-aminoimidazole. This amine is presumably identical with the aromatic amine accumulated by biotin-deficient yeast reported earlier by Chamberlain *et al.* (3).

Estes *et al.* (4) have studied a related system in which biotin appears to have a role in the interaction of ornithine and carbamyl phosphate to form citrulline by *Streptococcus lactis*. Williams & Cauthen (5) observed that biotinylphosphate was equivalent in activity to biotin in the deamination of aspartic acid by *Bacillus cadaveris*. Williams and associates (6) and Carlson & Whiteside-Carlson (7) have shown that biotin is essential for glucose dissimilation by *S. cerevisiae* and some species of leuconostoc. The results indicate that biotin functions in the hexokinase reaction as judged by the rate and extent of glucose utilization by biotin-deficient yeast (6). Whether biotin influences the synthesis of hexokinase or acts as a coenzyme was not clear from these experiments. As in many other studies with this vitamin, the addition of biotin to the cell-free extracts failed to reactivate the system obtained from the biotin-deficient yeast.

Other studies give some hints as to the role of biotin in the oxidation of C<sup>14</sup>-labelled acetate [Gram (8); Jacobsohn & Corley (9)] and with vitamin B<sub>6</sub> in histidine metabolism [Baldridge & Tourtelotte (10)].

In nutritional studies Jutton & Parsons (11) observed that rats fed raw egg white rations grew better and had higher liver biotin levels when cooked cornstarch was used as the carbohydrate in place of sucrose. A report by Quevedo (12) describes histological changes in the hair follicles of biotin-deficient mice and Gautier *et al.* (13) reported on a dermatitis condition attributable to a biotin deficiency in young boys. Three nutritional studies on biotin with microorganisms are of interest [Scheunert & Haenel (14); Ferguson & Lichstein (15); Hedgecock (16)]. In the last paper cited, it was reported that inhibition of *Mycobacterium tuberculosis* by *p*-aminosalicylic acid can be competitively reversed by *p*-aminobenzoic acid and noncompetitively by methionine and biotin. At high levels of *p*-aminosalicylic acid, certain amino acids, purines and fatty acids were required in addition to methionine and biotin for inhibition reversal. Ferguson & Lichstein studied various factors that influence the dose response curve of a biotinless mutant of *Escherichia coli*. Of interest is their observation that glucose additions spare the biotin requirement for this organism (15).

#### NICOTINAMIDE

The principal areas of biochemical study relate to the mechanism of the biosynthesis of tryptophan from indole and anthranilic acid by microorganisms, the metabolic conversion of tryptophan to kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and other nicotinic acid precursors, and the metabolism of nicotinic acid by animals and bacteria.

*Biosynthesis of tryptophan.*—It has been known for some time that anthranilic acid and indole are two major precursors in the biosynthesis of

tryptophan by *Lactobacillus arabinosus* and *E. coli* mutants. Studies by Gibson *et al.* (1) show that *E. coli* mutants accumulate indole on a medium of glucose and ammonium salts. The addition of serine stimulates the amount of indole accumulated. It is likely that serine in this case is functioning as a precursor of anthranilic acid and not in the conversion of anthranilic acid to indole. Earlier studies have shown that serine is also utilized in the conversion of indole to tryptophan.

The reports by Yanofsky (2) and Gots & Ross (3) are particularly significant in clarifying some of the intermediate steps in the conversion of anthranilic acid to indole. With the use of purified enzyme preparations from *E. coli* mutants it has now been established that anthranilic acid plus 5-phosphoribosyl-1-pyrophosphate forms indole-3-glycerol phosphate and this compound is then converted to indole plus triose phosphate. The mechanism of formation of indole glycerol phosphate appears to involve first, the formation of the ribotide of anthranilic acid, and then, by enolization, decarboxylation and ring closure, the pyrrole ring of indole is formed, according to Yanofsky (2). Other studies had shown that the carboxyl group of anthranilic acid is lost in this transformation and that the two carbons of the indole ring are probably derived from C-1 and C-2 of the ribose compound referred to above (4, 5). This reaction sequence in *E. coli* appears unique in that indole and serine are then utilized to form tryptophan, and the three carbon side chain of indole-3-glycerol phosphate is not utilized directly, even though this would appear quite feasible.

*Conversion of tryptophan to nicotinic acid.*—Research on the metabolic precursors of nicotinic acid continued to be very active during the past year. Perhaps most significant was the refinement of techniques for the separation and measurement of several of these metabolites, and increasing knowledge of the reactions involved in the conversion of 3-hydroxyanthranilic acid to quinolinic acid. The latter reaction has been of particular interest, since it involves the conversion of a benzene-type compound to a pyridine-type compound. Certain nutritional studies have also established the over-all efficiency of tryptophan as a nicotinic acid precursor in man and other animals.

The metabolic conversion of tryptophan to nicotinic acid may be considered to proceed as follows: tryptophan→kynurenine→3-hydroxykynurenine→3-hydroxyanthranilic acid→1-amino-4-formyl-1,3-butadiene-1,2-dicarboxylic acid→quinolinic acid→nicotinic acid.

By the use of chromatographic, spectrophotometric, and colorimetric methods, the amounts of various tryptophan metabolites excreted in the urine by normal and cancer patients before and after tryptophan feeding have been determined by Price & Brown (6), Boyland & Williams (7), Price & Dodge (8), Brown & Price (9), Takahashi & Price (10), Brown (11), and Vivian *et al.* (12). The metabolites determined include kynurenine, N-acetylkynurenine, kynurenic acid, xanthurenic acid, 8-methyl ether of xanthurenic acid, quinaldic acid, 8-hydroxyquinaldic acid, *o*-amino hippuric acid, anthranilic acid glucuronide, 3-hydroxykynurenine, 3-hydroxyanthranilic acid



and its sulfuric ester, and N-methyl-2-pyridone-5-carboxamide (pyridone).

It was found that acetyl-D-tryptophan ingestion by man did not increase the amount of these metabolites excreted. Acetyl-L-tryptophan ingestion raised the urinary excretion, but less than that observed for equimolar quantities of L-tryptophan or D-tryptophan (6). Langner & Edmonds (13) have also shown that acetyl-D-tryptophan is poorly absorbed. The detection of the 8-methyl ether of xanthurenic acid in urine was reported for the first time (8) and urinary excretion of this compound was shown to be increased by tryptophan administration. An interesting report that patients with bladder cancer excrete more of certain of these compounds (with or without additional tryptophan supplements) offers promise as an important approach in the study of the biochemical aspects of cancer (7). It has also been shown (14) that a significant proportion of mice develop bladder cancer following insertion of 3-hydroxyanthranilic acid pellets in the bladders.

The over-all metabolism of  $C^{14}$ -labelled tryptophan by the rat has provided additional quantitative data on the formation of quinolinic acid and other metabolic products (15). DL-Tryptophan labelled in positions 3a, 7a, and 7 was injected into rats maintained on a 9 per cent casein diet. The percentage of  $C^{14}$  injected, accounted for as respiratory  $CO_2$ , deposition in the tissues, and excretion in the urine and feces, was 25, 16 to 30, and 15, respectively, for periods up to 48 hr. after injection. Thus, about 25 per cent of the radioactivity was not accounted for by these measurements. Approximately one-half of the radioactivity in the urine was accounted for by the radioactivity of the quinolinic acid, urea, and N-methylnicotinamide excreted. Considerable quantities of other unidentified radioactive compounds were also present in the urine.

In confirmation of earlier nutrition work, tryptophan was shown to be a direct precursor of quinolinic acid, and the specific activities of the carboxyl carbon and nicotinic acid derived from quinolinic acid were in accord with the specific activities of the labelled carbons of the tryptophan injected. In other experiments by Henderson *et al.* (16), kynurenine and hydroxykynurenine were shown to be effective precursors of nicotinic acid in the rat.

Following the injection of carboxyl-labelled 3-hydroxyanthranilic acid into rats, the specific activities of the quinolinic acid and N-methylnicotinamide isolated from the urine underwent minimal dilution, as compared to the material injected (17). Relatively little radioactivity was found in picolinic acid [Mehler (18)], suggesting that this pathway of 3-hydroxyanthranilate metabolism was of minor importance under these circumstances. In these studies (17), and those referred to earlier with labelled tryptophan (15), relatively large quantities were rapidly metabolized to  $CO_2$  (25 to 60 per cent). The pathways for these oxidative steps are not known.

The significance of quinolinic acid as an intermediate in the biosynthesis of nicotinic acid from tryptophan continues to receive attention. In nutritional studies with rats and other animals, quinolinic acid has been found to be a less efficient source of nicotinic acid than its precursor, 3-hydroxyan-



thranilic acid. It is active, however, and because it is a dicarboxylic acid, cell permeability may complicate interpretation of the experiments. It is also active in supporting the growth of *Xanthomonas pruni*. The experiments with liver preparations *in vitro* show essentially complete conversion of 3-hydroxyanthranilic acid to quinolinic acid and the formation of nicotinic acid has not been detected with the methods used thus far. In experiments by Quagliariello & Pietra (19) chick embryos were capable of forming nicotinic acid from 3-hydroxyanthranilic acid at levels comparable to those observed earlier for tryptophan by Schweigert *et al.* (20), and quinolinic acid formation could not be detected. It should be pointed out, however, that if the rate of nicotinic acid formation from quinolinic acid was more rapid than the production of quinolinic acid from 3-hydroxyanthranilic acid, detection of quinolinic acid formation would not be feasible, in the absence of an appropriate inhibitor of the quinolinate→nicotinic acid reaction.

Findings by Wiss & Bettendorf (21) show that the primary product of the enzymatic cleavage of 3-hydroxyanthranilic acid by liver preparations is 1-amino-4-formyl-1,3-butadiene-1,2-dicarboxylic acid. This could arise by scission of the benzene ring in 3-hydroxyanthranilic acid between C-3 and C-4.

A new suggestion has been made by Harris & Binns (22) that 3-hydroxyanthranilic acid may be cleaved between C-2 and C-3 to form the hypothetical alpha-aminomethyl-trans-cis-muconic acid. After decarboxylation and ring closure, tetrahydronicotinic acid would be formed, which presumably could then be transformed into nicotinic acid. In support of this possibility, Harris and Binns have synthesized the trans-trans derivative of muconic acid and have shown this compound to be active for *X. pruni*.

It would appear that preparation of 3-hydroxyanthranilic acid labelled in specific carbon atoms would be most helpful in clarifying the significance of quinolinic acid as an intermediate in nicotinic acid formation *in vitro* and *in vivo* and in ascertaining the point of cleavage of 3-hydroxyanthranilate. Hanks & Segel (23) have utilized tritium-labelled quinolinic acid to show that 8 to 24 per cent of the N-methylnicotinamide excreted by rats arose from injected quinolinic acid. With doses of 5 and 20 mg., respectively, 95 and 68 per cent of the injected quinolinic acid was excreted unchanged.

Extensive studies by Horwitt and co-workers (24) show that approximately 60 mg. of tryptophan is equivalent in activity to 1 mg. of nicotinic acid for man. This efficiency of conversion correlates very well with earlier data showing that 1 to 2 per cent of the tryptophan ingested with adequate diets is excreted as nicotinic acid metabolites in the urine (principally N-methylnicotinamide and the pyridone). It is of interest that Horwitt *et al.* reported that the tryptophan of lactalbumin was as well utilized as L-tryptophan for nicotinic acid synthesis. Similar complete utilization of tryptophan from proteins for protein synthesis has been observed by Lushbough *et al.* (25) and Gupta & Elvehjem (26). Banerjee & Basak have shown that tryptophan is an effective precursor of nicotinic acid in the Rhesus monkey.

Ascorbic acid deficiency does not affect this conversion, as judged by the levels of nicotinic acid derivatives excreted following tryptophan supplementation (27).

*Metabolism of nicotinic acid.*—The metabolism of carboxyl-labelled nicotinamide by the chick was investigated by Chang & Johnson (28). These workers showed that seven  $C^{14}$ -labelled derivatives were excreted, including nicotinic acid and dinicotinyl ornithine, in confirmation of earlier studies. Two derivatives (beta-nicotinyl-D-glucuronide and nicotinuric acid) that were not known to be excreted by the chick, and two new metabolites of nicotinic acid (delta-nicotinyl ornithine and alpha-nicotinyl ornithine) were also detected. No methylated derivatives were observed for this species. Sundaram & Sarma have observed that pantothenic acid-deficient rats excrete lower quantities of certain nicotinic acid metabolites than the controls (29).

The fermentation of nicotinic acid by an anaerobic microorganism isolated by the enrichment culture technique has been investigated by Harary (30, 31). This organism is capable of utilizing nicotinic acid as a source of energy, nitrogen and carbon for growth. With a yeast extract and peptone medium, nicotinic acid is required for growth. With the use of cell suspensions, one mole of nicotinic acid is oxidized to one mole each of acetate, propionate, ammonia, and  $CO_2$ . Pyridine is not oxidized, suggesting that decarboxylation is not the first step in the breakdown of nicotinic acid. Further study showed that 6-hydroxynicotinic acid could be detected in anaerobic systems in the presence of methylene blue. The formation of this compound presumably is the first step in the breakdown of nicotinic acid. Harary suggested that the hydroxy compound may be oxidized further to the pyridone derivative.

The detection of 6-hydroxynicotinic acid as the initial product of nicotinic acid breakdown confirms an earlier report [Hughes (32)] on the hydroxylation of nicotinic acid by pseudomonas. In this, and a subsequent study with the use of  $O^{18}$  by Hunt *et al.* (33), it was shown that the oxygen of the hydroxyl group was derived from  $H_2O$  and not from  $O_2$ . These hydroxylation pathways appear to differ from those reported for the formation of 5-hydroxytryptophan from tryptophan by *Chromobacterium violaceum* [Mitoma *et al.* (34)] and 3-hydroxykynurenine from kynurenine by liver mitochondria [DeCastro *et al.* (35)]. A report by Niemer & Oberdorfer (36) shows that 5-hydroxyanthranilic acid stimulates growth of a strain of *E. coli* under conditions in which 3-hydroxyanthranilic acid and anthranilic acid are ineffective. Mirsky *et al.* (37) reported that 5-hydroxytryptophan and several other tryptophan metabolites inhibit the action of insulinase by rat liver preparations.

In view of these significant findings, we may expect expanded interest in the metabolism of the hydroxy derivatives of nicotinic acid and related compounds.

*Stability of nicotinic acid.*—Additional reports extend earlier knowledge on the high stability of nicotinic acid in foods or in pure solutions when ex-

posed to various storage, irradiation, and heat treatments [Westerman *et al.* (38); Day *et al.* (39); and Thomas & Calloway (40)]. Approximately 50 per cent of the nicotinic acid was destroyed, however, when exposed to ethylene oxide in studies by Bakerman *et al.* (41). An increase in utilization of nicotinic acid from cooked as compared to raw maize (rat) and by alkaline treatment of maize (pig) has been reported by Pearson *et al.* (42) and Kodicek *et al.* (43). These and earlier studies of a similar nature were designed to elucidate the chemical nature of the so-called "bound, nonutilizable" forms of nicotinic acid in unprocessed corn.

An interesting concept regarding the stability of the metabolically active form of nicotinic acid, diphosphopyridine nucleotide (DPN) in relation to serological types of streptococcus has been studied by Carlson *et al.* (44), Bernheimer *et al.* (45) and Lazarides & Bernheimer (46). In this work the activity of DPNase (which splits the ribose—nicotinamide bond) was correlated with the serological type. Within 235 strains representing nine serological types of Group A streptococci summarized by Lazarides & Bernheimer (46), only one of 120 strains, representing five types, produced DPNase, while 108 of 115 strains, representing the four other types, produced DPNase. These findings suggest that there may be some relationship between blocking of DPN-linked enzymes (due to DPNase action) and the capacity to kill leucocytes in certain types of streptococci.

*Nicotinic acid antagonists.*—The effects of 6-amino nicotinamide and 2-ethylamino-1,3,4-thiadiazole on certain cancer conditions have been studied by Shapiro *et al.* (47), Baserga *et al.* (48) and Halliday *et al.* (49). The 6-aminonicotinamide is quite effective as a nicotinic acid antagonist, and is seven times as active as the acid. When 15 to 30 mg. are added per kg. of diet, deficiency symptoms occur. Approximately 150 to 300 mg. of nicotinamide are required to alleviate these symptoms. The 6-amino derivative is a tumor inhibitor (49).

#### VITAMIN B<sub>6</sub>

*Methods and chemistry.*—During the past year, Fasella *et al.* (1) reported further studies on the chemical reactions between amino acids and pyridoxal or pyridoxal phosphate, and alpha-keto acids and pyridoxamine or pyridoxamine phosphate. With copper as chelating agent, they isolated two strongly fluorescent intermediates in the transamination reactions, and identified them as derivatives of Schiff base type (imine) compounds between pyridoxal and, in this case, alanine, and of pyridoxamine and pyruvate.

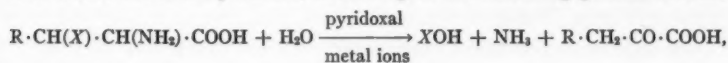
Matsuo (2) reported that these transamination reactions between the -al and amino acid, or the -amine and alpha-keto acid also occur readily in absolute ethanol solution. Neither metallic salts nor elevated temperatures were required, and absorption spectra indicated that the reactions proceed via Schiff base intermediates. Thus, transamination may occur nonenzymatically in nonaqueous solutions when catalyzed by pyridoxal or -amine, duplicating reactions catalyzed *in vivo* by transaminases.

Matsuo (3) also evaluated the stability constants of the Schiff base compounds formed between pyridoxal phosphate and several amino compounds in aqueous solution. Spectrophotometric evidence for the chelation of these compounds with  $\text{Co}^{++}$ ,  $\text{Al}^{++}$ ,  $\text{Cu}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Ni}^{++}$  and  $\text{Mn}^{++}$  was reported, and the metal ions could be removed from these chelate complexes (metal-pyridoxal phosphate-amino acid) using suitable concentrations of ethylenediaminetetraacetic acid or 2,3-dimercaptopropanol.

Metzler (4) reported that the formation of imines (Schiff base) in aqueous solutions of pyridoxal and amino acids occurs over a wide pH range. Maximum absorption at neutral pH was at about 414 m $\mu$ , with a shift to 365 m $\mu$  at high pH levels. This author found that the imines formed have a pK of approximately 10.5, and that the presence of a beta-methyl group in the amino acid side chain increased, while an alpha-methyl group decreased, the stability of the imine compounds observed.

Longenecker & Snell (5) reported the activities of several different metal ions in catalyzing reactions of pyridoxal and amino acids, and found good correlation between the relative activities observed, and the order of the stability constants of the chelate complexes formed. Thus, chelation with the reactants appears to be a requirement for the catalysis of the transamination and cleavage reactions studied.

These authors (6) also reported further studies on nonenzymatic reactions catalyzed by pyridoxal phosphate, paralleling enzymatic reactions which are known to occur, and which require pyridoxal phosphate coenzyme activation. Using esters of serine and threonine, they found that several alpha, beta-elimination reactions occur rapidly in the presence of pyridoxal and one of several metal ions ( $\text{Cu}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Fe}^{+++}$ ,  $\text{Ga}^{+++}$  or  $\text{Al}^{+++}$ ), at or above room temperature, according to the following general scheme:



where X may be OH, Cl, Br,  $\text{OPO}_3\text{H}_2$ , etc. It would thus appear from these and earlier studies that reactions *in vivo* involving pyridoxal coenzymes may proceed in a manner similar to the *in vitro* reactions which have been studied.

Wiegand (7) reported a study of the reaction of pyridoxal-5-phosphate with a series of hydrazides to form the hydrazones, and showed the acid dissociation constants to be correlated linearly with the specific reaction rates. This work may be of especial importance in view of the pharmacological properties of isonicotinyl hydrazide and its therapeutic uses, and the earlier reports which have shown that hydrazides inhibit several enzyme systems in which pyridoxal phosphate is the active coenzyme (8).

Heyl *et al.* (9) synthesized the pyridoxine isomer 2,5-bis-(hydroxymethyl)-3-hydroxy-4-methylpyridine and reported that it possessed no vitamin B<sub>6</sub> activity for the rat.

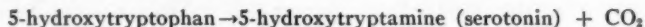
Parrish *et al.* (10) reported further studies on the microbiological yeast assay method for vitamin B<sub>6</sub> using *Saccharomyces carlsbergensis* (A.T.C.C.

9080) as the test organism. Optimal growth response was obtained when the pH of the medium was adjusted to 3.5 to 4.5, considerably lower than the levels previously recommended. Under these conditions, with cystine present in excess, the growth response to pyridoxine and pyridoxal were equal, while reduced growth was observed for the amine form of the vitamin.

*Vitamin B<sub>6</sub> enzymes and metabolism.*—Beechey & Happold (11) reported the isolation of a  $Mg^{++}$ -activated enzyme catalyzing the reversible interconversion of pyridoxal phosphate and pyridoxamine phosphate from *E. coli*. Pogell (12) reported a new aerobic pathway for conversion of pyridoxamine phosphate to the -al phosphate by the action of extracts from the liver of rat, rabbit, or calf on pyridoxamine phosphate, without other cofactor requirements. In contrast to the *E. coli* enzyme system (11), this conversion did not appear to be readily reversible. Baranowski *et al.* (13) have isolated pyridoxal-5-phosphate from crystalline muscle phosphorylase. The functional significance of the pyridoxal-5-phosphate in the enzyme system has not yet been clarified, however.

Schreier *et al.* (14) reported lowered catabolism of  $C^{14}$ -labelled glutamic acid in  $B_6$ -deficient rats, with the interesting result that incorporation of the amino acid into tissue and organ proteins was increased under these conditions. Administration of pyridoxal phosphate accelerated catabolism of glutamate in the deficient animals as expected. Baldrige & Tourtelotte (15) studied the effect of vitamin  $B_6$  deficiency on histidine metabolism in the rat and found that the levels of aspartic-glutamic transaminase decreased and histidase increased, while urocanase and rhodanese levels were unaffected by the deficiency.

Weissbach *et al.* (16) reported the isolation and function of pyridoxal phosphate as the active coenzyme for 5-hydroxytryptophan decarboxylase, which catalyzes the reaction:



Tissue serotonin levels were diminished in  $B_6$ -deficient chicks, and the rate of conversion of 5-hydroxytryptophan to serotonin was also reduced in the deficient animals. Semicarbazide administration gave diminished conversion *in vivo* in the mouse, but no effect was noted in the guinea pig.

A report by Yamada *et al.* (17) indicates that pyridoxal phosphate is required for the synthesis of pantetheine, presumably in the decarboxylation of pantothenyl cysteine. It is not required for the further conversion of pantetheine to coenzyme A, however. Schulman & Richert (18) reported that the rate of heme synthesis observed in the red cells of  $B_6$ -deficient ducklings was appreciably reduced *in vitro*. Pyridoxal phosphate or pyridoxamine phosphate addition *in vitro* stimulated heme synthesis from glycine and succinate, while pyridoxine, -al, and -amine did not.

Blaschko & Hope (19) and Hope (20) found that  $B_6$ -deficient hooded rats excrete L-cystathionine in the urine, while similar albino rats do not. They suggest that the cystathionine-cleaving enzyme is lost from the tissues

at a more rapid rate than the enzyme required for cystathionine formation. Both enzymes require pyridoxal phosphate for these trans-sulfuration reactions.

*Vitamin B<sub>6</sub> in human nutrition and metabolism.*—Bessey *et al.* (21) have completed a study of nine infants 1 to 12 months old suffering convulsions due to inapparent cause. These authors suggest that the vitamin B<sub>6</sub> requirements of these infants were above average, and that therapeutic doses of 5 to 10 mg. pyridoxine hydrochloride daily should be given to infants exhibiting such symptoms. "Normal" requirements are thought not to exceed 0.3 mg. B<sub>6</sub>. Hunt suggested (22) that congenital abnormalities in vitamin B<sub>6</sub> metabolism may be observed in cases where the dietary intake of B<sub>6</sub> would usually be considered adequate. In such instances, B<sub>6</sub> intakes 10 to 15 times as great may be required to prevent convulsions, even though xanthurenic acid excretion following tryptophan load tests may appear normal at the normal intake levels. In the adult, metabolic irregularities have been reported which apparently result in a B<sub>6</sub>-anemia, which also responds to large doses of the vitamin [Harris *et al.* (23)].

Girdwood (24) reported that in 10 cases of idiopathic steatorrhea and related cases exhibiting impaired absorption of fats and fatty acids, no impairment of the absorption of pyridoxine could be demonstrated.

*Animal nutrition and metabolism.*—Williams & Cohen (25) reported that when the protein/energy ratio of experimental rations was held constant, increases in the fat level from 5 to 40 per cent had no significant effect in ameliorating the pyridoxine deficiency symptoms observed, indicating that the B<sub>6</sub> requirement is related to the amount of protein to be metabolized, and that feeding fat does not spare vitamin B<sub>6</sub> *per se*. Guggenheim & Diamant (26) reported that young pyridoxine-deficient rats had enlarged adrenals with normal cholesterol content, exhibited increased glucose utilization in muscle tissues, and found no deviation from normal metabolism of carbohydrate which could be attributed to adrenal insufficiency.

Moldave (27) found that following injection of DL-phenylalanine-3-C<sup>14</sup>, tissues of control animals all exhibited markedly higher C<sup>14</sup> activity compared to the tissues of B<sub>6</sub>-deficient rats. Three consecutive daily doses of 1 mg. pyridoxine hydrochloride prior to injection gave no significant response in the control animals, while the B<sub>6</sub>-deficient animals then showed tissue radioactivity levels approximating that of the controls, suggesting that the deficiency had been substantially eradicated. Using carboxyl-labelled alpha-aminoisobutyric acid, which is actively transferred but neither degraded nor anabolized, Riggs *et al.* (28) demonstrated significantly increased liver and serum activity levels, while skeletal muscle, heart, kidney and duodenum levels were all normal or below normal, suggesting impaired transport in B<sub>6</sub>-deficient rats. Simultaneous injection of 1 mg. pyridoxine hydrochloride with the test compound reversed the effects observed in the deficient animals.

Ross & Pike (29) reported that depletion of maternal stores of vitamin B<sub>6</sub> during pregnancy leads to changes in serum protein and non-protein nitro-



gen concentrations similar to those reported for the toxemias of pregnancy in the human female. These authors (30) also observed maternal weight gains of less than 100 gm., average fetal weights of less than 5 gm., and increased incidence of fetal resorption under these conditions. Pike & Brown (31) found that during pregnancy, the hemoglobin and hematocrit values of vitamin B<sub>6</sub>-deficient animals fall, and plasma protein concentrations increase during the first two weeks, then fall rapidly during the final week of pregnancy.

Bergeret & Chatagner (32) reported that vitamin B<sub>6</sub> deficiency in the rat caused a substantial decrease in the urinary excretion of taurine, accompanied by a decrease in the taurine conjugates and a large increase in the glycine conjugates in the bile. No over-all change in bile acid production occurred, and the maintenance of significant levels of taurine conjugates in B<sub>6</sub>-deficient rats' bile suggested that an alternative synthetic pathway for taurine formation, other than decarboxylation of sulfated amino acids, may be available to the animal. Combridge (33) also reported increased adrenal weights in B<sub>6</sub>-deficient rats (cf. 26) as well as lower thymus weights and unaffected spleens. The histological changes observed in the adrenal and thymus glands of the deficient animals preceded the fatty infiltration of the centrilobular region of the liver, and the author suggests that hyperactivity of the *zona fasciculata* may have caused the changes in liver and thymus observed.

The multiplication of tubercle bacteria has been reported to be substantially greater in rats deprived of vitamin B<sub>6</sub> than in animals fed a complete regimen, following inoculation with the pathogens [Charconnet-Harding and Hirsch (34)].

Beaton *et al.* (35) reported that the food intake of B<sub>6</sub>-deprived rats can be increased by administering insulin, and that under these conditions the vitamin B<sub>6</sub> deficiency does not appear to impair fat deposition *per se* in the rat. Alanine-glutamic transaminase activity in the liver decreased under these conditions, as did aspartic-glutamic transaminase activity. These changes appear to be a specific effect of the deficiency.

Hove & Herndon (36) reported the production of vitamin B<sub>6</sub> deficiency in the rabbit, with the following symptoms: scaly skin of the ears, mild anemia, convulsion, increased blood clotting time, and marked creatinuria with paralytic collapse and death. Requirements of the rabbit were estimated to approximate 1 µg./gm. of diet, as measured by growth response. Miller *et al.* reported that the pyridoxine requirement of the baby pig is greater than 0.75 mg. but probably less 1.0 mg./kg. of solids in the ration (37).

Current research by Greenberg & Moon (38) and Mushett & Emerson (39) on the relationship between vitamin B<sub>6</sub> deficiency and the development of atherosclerotic lesions in monkeys and dogs suggests that insufficient supplies of the vitamin may result in conditions of stress which tend to accelerate the development of the disease in these animals. Massive doses of pyridoxine fed to deficient animals may have some beneficial effects, but



such treatment has not brought about a complete reversal of the lesions observed in the animals tested to date.

*Microorganisms.*—Yano (40) and Yano & Jujita (41) reported that in humans, the total quantities of pyridoxine, -al and -amine and 4-pyridoxic acid excreted in urine and feces, and the differences between excreted and ingested vitamin B<sub>6</sub> were all markedly increased when vegetable diets replaced ordinary mixed diets. Addition of cellulose to the vegetable diet resulted in further increases. Changing from the mixed diet to a meat diet resulted in decreased levels, which were, however, reversed and increases observed with the addition of cellulose to the meat diet.

Strode (42) studied the relations between the diet fed to 0 to 3-month-old calves, the intestinal microflora, and fecal excretion of vitamin B<sub>6</sub>. He found that the fecal excretion increased throughout the three-month experimental period, in spite of the shift at one month from a milk diet (6 mg. B<sub>6</sub>/day) to a mixed feed containing about half as much vitamin B<sub>6</sub> daily. When grown on a synthetic salt medium, all of the microorganisms isolated from the feces produced B<sub>6</sub> in the medium, with *E. coli*, *E. coli* var. *communior* and the enterococci most effective, suggesting that substantial amounts of vitamin B<sub>6</sub> are made available to the calf by intestinal microorganisms. Thus, these findings are in accord with those reported by Yano & Jujita (40, 41) and it would appear that under appropriate conditions, important portions of the vitamin B<sub>6</sub> requirements may be provided by microbial synthesis in the intestine.

Holden & Holman (43) reported that in vitamin B<sub>6</sub> deficiency, *L. arabinosus* 17-5 and *Streptococcus faecalis* exhibit reduced ability to accumulate glutamic acid from buffered media, and that morphological and metabolic differences between B<sub>6</sub>-deficient and -adequate cells may account for this observation. They report that the liberation by washed cells in buffer solution, of substances with absorption maxima at about 260 mμ is increased three- to tenfold in the B<sub>6</sub>-deficient cells.

Kleinschmidt *et al.* (44) reported that purified typhus rickettsiae contain substantial amounts of vitamin B<sub>6</sub>, as well as nicotinamide, thiamine and riboflavin, while containing relatively small amounts of pantothenic acid, folic acid, biotin, and cyanocobalamin. Tobacco mosaic virus was found to contain very small amounts of nicotinamide, pantothenic acid, biotin and cyanocobalamin while coliphage T<sub>6</sub> contained small amounts of nicotinamide. Neither of the latter appeared to possess vitamin B<sub>6</sub> activity. These findings appear consistent with the known independent enzyme activity of the rickettsiae, and the absence of such activity in viruses.

*Vitamin B<sub>6</sub> analogues and antagonists.*—Kuchinskas & du Vigneaud (45) have shown that the addition of L-penicillamine to the diet of the albino rat results in increased requirements for vitamin B<sub>6</sub>, and results in gross symptomatology similar to that observed in B<sub>6</sub>-deficient animals. The effect may be due to competitive inhibition, since administration of increased amounts of pyridoxine, -al or -amine overcame the effects observed.

Ross & Pike (30, 31) reported that nitrogen retention levels observed for rats fed deoxypyridoxine-supplemented rations were significantly reduced when compared to the vitamin B<sub>6</sub>-deficient animals, indicating further reduction in the efficiency of handling dietary protein due to the deoxypyridoxine supplement, and intensification of the vitamin B<sub>6</sub> deficiency.

Dietrich & Borries (46) pointed out that although deoxypyridoxine inhibits glutamic-aspartic transaminase activity, cysteine desulfhydrase activity is unaltered by this antagonist, while at the same time it is very sensitive to the depletion of body stores of vitamin B<sub>6</sub>. These workers found that apo-cysteine desulfhydrase has little affinity for deoxypyridoxine phosphate *in vitro*, with concentrations of deoxypyridoxine phosphate:pyridoxal phosphate of 10:1, while under similar conditions, apo-tyrosine decarboxylase from *E. coli* is about 35 per cent inhibited and pig heart apo-transaminase is 100 per cent inhibited.

Shintani (47) and Torigoe & Kinoshita (48) reported that toxopyrimidine showed antivitamin B<sub>6</sub> activity similar to that of deoxypyridoxine. This antagonist caused death in B<sub>6</sub>-deficient mice, and resulted in reduced gains in weight of animals on a basal ration. These effects could be overcome by increasing the intake of vitamin B<sub>6</sub>, as in the case of deoxypyridoxine.

Haenel & Meyer (49) and Sakuragi & Kummerow (50) found in independent investigations that pyrimin (2-methyl-4-amino-5-hydroxymethyl-pyrimidine) is a vitamin B<sub>6</sub> antagonist for certain micro-organisms. It was found to act as a competitive inhibitor for *E. coli*, *S. carlsbergensis* and *Neurospora sitophila* 299, while in the case of *S. faecalis*, the inhibition observed was non-competitive. The work is of especial interest since pyrimin is the pyrimidine fragment of thiamine.

The latter authors (50) synthesized several analogous derivatives in which the pyrimidine portion of the thiamine molecule was supplanted by vitamin B<sub>6</sub>. They reported that the condensate of 4-methyl-5-(beta-hydroxy-ethyl) thiazole and 5-monobromopyridoxamine gave growth depression in *Lactobacillus fermenti* 36 (A.T.C.C. 9338) which was not reversed by thiamine or its pyrophosphate, but could be reversed by certain sulfhydryl compounds, including D- and L-cysteine, L-homocysteine, reduced glutathione and thioglycolic acid. The condensate of the thiazole and 5-monobromo-4-deoxypyridoxine did not inhibit growth of this organism.

*Vitamin B<sub>6</sub> in foods.*—Day *et al.* (51) reported that gamma irradiation of raw ground beef ( $3 \times 10^6$  roentgen equivalents physical) destroyed approximately 25 per cent of the vitamin B<sub>6</sub> activity. These authors also found that supplementation of diets, marginal in pyridoxine, with penicillin gave increased growth rates and liver levels of vitamin B<sub>6</sub>. Comparable results for the vitamin B<sub>6</sub> content of beef were obtained using a microbiological assay, with *S. carlsbergensis* (A.T.C.C. 4228) as the test organism, and with rat growth bioassays.

Hodson (52) found that pyridoxal is partially converted to pyridoxamine during the sterilization of evaporated milk, and suggested that this conver-

sion continues during storage. A part of the vitamin B<sub>6</sub> activity also appeared to be converted during storage to an unknown form having activity equal to pyridoxine for *N. sitophila* 299, much less activity for *S. carlsbergensis* and *Lactobacillus casei*, and still less for the rat. This author reported that although *N. sitophila* 299 and *S. carlsbergensis* give similar results for vitamin B<sub>6</sub> activity for fresh, pasteurized, and nonfat dry milk, the former method gives higher results than the latter when applied to evaporated milk.

Witting *et al.* (53) reported that in feeding autoxidatively polymerized, thermally autoxidatively polymerized or thermally polymerized fats to rats, supplementation of the diet with pyridoxine enhanced the apparent nutritional value of the fats fed, especially at the lower protein levels tested.

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## WATER-SOLUBLE VITAMINS, PART III<sup>1,2</sup>

### THIAMINE, LIPOIC ACID (THIOCTIC ACID), PANTOTHENIC ACID, RIBOFLAVIN, AND INOSITOL

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#### THIAMINE

Studies on bacterial synthesis and destruction of thiamine have been reviewed by Hayashi (1). Bacteria which secrete potent thiaminases have been isolated from human feces in patients suffering from "thiaminase disease." The bacterial enzymes appear to be responsible for the thiamine deficiency found in these subjects, since orally administered thiamine is decomposed by the enzymes in the digestive tract. Thiaminase has been found in the contents of the entire distal portion of the intestinal tract in autopsy material from patients with previous evidence of thiaminase disease (2). The feces of healthy subjects did not break down thiamine (3). Thiaminase diseased patients with beriberi symptoms and low blood thiamine levels are often found in the Japanese clinics. They can be cured by oral administration of homosulfanilamide or antibiotics followed by preparations of lactic acid bacteria. Bacteria which have been isolated from feces of subjects with thiaminase disease include *Bacillus thiaminolyticus* Matsukawa et Misawa, *Bacillus aneurinolyticus* Kimura et Aoyama, and *Clostridium thiaminolyticus* Kimura et Liao (1). A thiamine-destroying fungus, *Trichosporon aneurinolyticum*, has also been isolated from the mouth of one subject (4).

Healthy human subjects were found to be resistant to infection by *B. thiaminolyticus*, whereas subjects with predominantly putrefactive bacteria in the intestine easily became carriers (5). Sheep and rabbits were quite resistant, whereas resistance decreased in the hen, rat, guinea pig and cat, respectively (6). Spontaneous thiaminase disease is also found in cats without experimentally administering the bacillus. Hamada (7) has shown that various bile acids differ in their inhibition of growth of this organism, and has suggested that differences in amounts and types of bile acids may account for differences in individual and species susceptibility. *B. aneurinolyticus* has also been isolated from the feces of chicks showing signs of motor paralysis of the extremities, indicative of thiamine deficiency (8). Symptoms

<sup>1</sup> This review covers the papers that were available to the authors up to November 1, 1957.

<sup>2</sup> The following abbreviations are used: ACTH for adrenocorticotrophic hormone; ATP for adenosine triphosphate; CoA for coenzyme A; CoASH for reduced coenzyme A; DPN and DPNH for the oxidized and reduced forms of diphosphopyridine nucleotide; FAD for flavin-adenine-dinucleotide; FMN for riboflavin-5'-phosphate; TPN and TPNH for the oxidized and reduced forms of triphosphopyridine nucleotide; and TPP for thiamine pyrophosphate or cocarboxylase.

of thiamine deficiency in hens were also observed following daily administration of *B. thiaminolyticus* (9). The reviewers suggest that stimulation of animal growth by addition of antibiotics to the diet may be due in part to suppression of growth of bacteria which destroy thiamine or other vitamins.

The decomposition of thiamine by *B. thiaminolyticus* is markedly increased by bases such as pyridine and aniline, producing heteropyrithiamine and anilinothiamine, respectively, by a base exchange reaction (3, 10). The activation of this bacterial enzyme by aromatic amines and heterocyclic compounds is similar to that of shellfish and fish thiaminases (11). From the action of crude *thiaminolyticus* thiaminase on thiamine, Murata & Morita (12) isolated a crude crystalline compound, probably consisting of the pyrimidine moiety of thiamine and a base of unknown structure. The compound was able to support growth of *aneurinolyticus* as effectively as thiamine. The degradation of thiamine by sulfurous acid has also been shown to be accelerated by amines (13). This is being studied as a possible model system for elucidation of the mode of action of *thiaminolyticus* thiaminase (14). Diacetylthiamine was decomposed by fern and shellfish thiaminases but not by the bacterial thiaminases (15).

The thiaminase produced by *aneurinolyticus* has been reported to differ from the other thiaminases, in that aromatic amines do not activate, and may even inhibit the enzyme (16). Thiamine is decomposed into pyrimidine and thiazole moieties which have been isolated and identified (16, 17). The pyrimidine portion does not couple with any of the amines tested, except aniline, as shown by isolation of anilinothiamine (18).

Kupstas & Hennessy (19) have shown that the structure of ichtiamin, formed from thiamine by clam extracts, is 4-amino-5-(2-amino-ethanesulfonyl)-methyl-2-methylpyrimidine, and that hypotaaurine (2-aminoethanesulfinic acid) is the precursor of the aliphatic side chain. Thiamine deficiency, reported in cats fed canned commercial cat food containing ground whole fish (20), appears to be due to destruction of thiamine by fish thiaminases during processing.

Kenten (21) has purified the thermolabile thiaminase from bracken fern, *Pteridium aquilinum* (L.) Kuhn, and has shown that in the presence of thiamine and pyridine, it catalyzes a transfer reaction with the formation of heteropyrithiamine. The differences in properties of the thermostable and thermolabile thiamine-decomposing factors of ferns have been summarized by Fujita *et al.* (22), who have separated the factors and studied the properties of each. Flavonoids, phenols, quinones, catechol derivatives and other compounds have thiamine-decomposing activity, similar to that of the naturally occurring thermostable thiamine-decomposing factors (23, 24). In ferns, the thiamine-decomposing activity appears to be due to flavonoids (24), and in sweet potato leaves, the active flavonoid has been isolated and identified as isoquercitrin (25).

Scott & Griffith (26) have compared the effects of dietary fat, protein, penicillin and ascorbic acid, separately and in combinations, in increasing growth and thiamine storage in rats receiving different levels of thiamine in

the diet. The four substances differed in their effects, and combinations were found to be more effective than the individual substances. Scott & Griffith postulate that the substances tested probably influence metabolism and intestinal flora. As suggested in the case of antibiotics, a possible role for these factors may be the suppression of growth of thiaminase-secreting bacteria. Thiamine-sparing effects of ascorbic acid in the rat have also been shown by increased survival time of thiamine-deficient rats (27) and by increased weight gains and higher thiamine levels in the liver (28). Mameesh *et al.* (29) reported that penicillin improved the growth of rats given sub-optimal levels of thiamine, but did not significantly alter the requirement for maximum growth on the diet used. Johansson (30) found that preparations of penicillin G and V increased weight gain significantly when given orally to rats on a thiamine-free diet, and to a lesser extent when given parenterally. Liver thiamine was increased, but data on the thiamine content of ileal and cecal contents were inconclusive. Synthesis of thiamine in the intestine of the rat was increased by addition of banana to a poor quality rice diet (31), and by addition of milk curds to the diet (32).

Rats on a carbohydrate-free diet devoid of thiamine are known to gain weight and survive for several months, whereas addition of as little as 5 to 10 per cent carbohydrate to the diet leads to polyneuritis and death (33). Addition of up to 20 per cent sorbitol to the thiamine-free diet containing no carbohydrate or as much as 40 per cent carbohydrate permitted continued weight gain and good survival (34). Good growth has also been obtained on diets containing sorbitol and carbohydrate, with all of the B vitamins omitted from the diet. The effects of sorbitol are presumably due to enhanced intestinal synthesis of thiamine and other vitamins, as evidenced by marked enlargement of the ceca. It would be of interest to know whether similar results can be obtained in the rat without access to fecal material.

In sheep fed eight rations, the concentration of thiamine in the dry matter of the intestinal contents was related to the thiamine content of the diet and decreased from the duodenum to the ileum (35). Almost all strains of *Lactobacillus bifidus* isolated from stools of infants (on breast feeding or mixed diets) and adults were found to synthesize considerable amounts of thiamine, whereas many cultures of *Lactobacillus acidophilus* synthesized very little thiamine (36). The addition of cellulose to either a meat or vegetable diet appeared to increase intestinal synthesis of thiamine in man, as determined by increases in fecal thiamine (37).

Kuboyama (38) found no change in the total thiamine content of hens' eggs during the course of incubation but there was a gradual transfer of esterified thiamine to the embryo during development. Howes & Hutt (39) confirmed the finding of different levels of thiamine in eggs of different breeds of hens but found no significant difference among various strains of each breed. Eggs taken from the same birds a month apart showed a remarkable consistency in thiamine values.

Brožek (40) reported the results of a comprehensive study of the psycho-

logical changes associated with acute thiamine deficiency in man. Biochemical data on thiamine excretion and plasma pyruvate levels were also presented. Subjects were maintained on 3300 calories per day with three levels of thiamine intake (0.6, 1.0, and 1.8 mg. per day) for 168 days, prior to acute deprivation on a thiamine-free diet for 15 to 27 days, and a final period of thiamine supplementation (5 mg. per day) for 9 to 12 days. In acute deprivation, the rate of onset of subjective and objective signs of deficiency reflected the previous level of thiamine restriction. Earliest symptoms included anorexia, nausea and vomiting. Thorough neuropsychiatric and psychological tests showed neurasthenia, marked emotional deterioration, lowering of pressure-pain threshold, impaired coordination, and increments in the psychoneurotic scales. No effect on performance in intelligence tests was observed. All of the symptoms responded rapidly and dramatically to thiamine supplementation.

North & Sinclair (41) found no evidence of peripheral nerve degeneration in rats repeatedly subjected to acute thiamine deficiency, even with concomitant pantothenate deficiency. Techniques of histological examination are discussed to account for disagreements with results of previous workers in studies on acute thiamine deficiency.

Ferrari (42) has measured the levels of several free amino acids in the brains of thiamine-deficient rats and in pair-fed and *ad libitum* fed controls. Using two dimensional paper chromatography, he found a significant decrease in glutamic acid content (about 15 per cent) in the thiamine-deficient animals. No differences were found in levels of aspartic acid, glutamine, or the other amino acids measured.

In electrocardiograms made weekly on pigs maintained on a thiamine-deficient diet from birth through five weeks of age, statistically significant increases in PR time, QT time and cycle length were observed, as contrasted with findings in control animals (43). Evidence of cardiac hypertrophy was also found in deficient animals. Changes in electrocardiograms and increases in heart choline ester content, which were found in thiamine-deficient rats, were shown to be only partly due to inanition (44).

Machida (45) reported that the concentration of insulin-like substances in the serum of mice was distinctly decreased in thiamine deficiency. Administration of thiamine to the thiamine-deficient animals raised the serum concentration of insulin to normal in one hour. *In vitro* addition of thiamine to mouse liver reduced liver insulinase activity, but administration *in vivo* had no such effect.

The weight responses of adult rats maintained on diets with various limiting levels of thiamine did not differ significantly at 5° or 25 to 28°C. (46). However, the data do not clearly establish the relationship between thiamine requirements at these temperatures.

The incorporation and distribution of S<sup>35</sup>-labeled thiamine in the liver was not altered by thiamine deficiency (47). Specific activity of the mitochondrial fraction was not greater than that of the other fractions. Turner *et al.* (48) have shown that relatively large amounts of thiamine speed the dextriniza-

tion of corn starch by salivary amylase *in vitro*. They associate "rapid dextrinization" rates in children with caries resistance and slower rates with proneness to dental decay. Limiting concentrations of nicotinic acid in cultures of *Proteus vulgaris* led to an accumulation of pyruvate, resulting in gradual deficiency of thiamine (49). Thiamine also plays an important role in the formation of ethyl acetate by the yeast *Hansenula anomala* (50). Pine (51) has shown that, in the yeast phase, eight of eleven strains of *Histoplasma capsulatum* require thiamine for maximum growth at 37°C.

In a study of the stability of several coenzymes in hemolyzed reticulocytes and red cells of the rabbit, it was shown that TPP and TPN underwent slow cleavage, whereas FAD and CoA were stable (52). From measurements in ten hyperthyroid patients and four control subjects, Giarnieri & Ipata (53) report that blood levels of TPP are reduced and those of FAD increased in hyperthyroidism. Kiessling (54) studied the incorporation of radioactive phosphate into thiamine phosphates in yeast and found a greater degree of incorporation into thiamine triphosphate than into the mono- or diphosphate. The radioactivity in the triphosphate was localized mainly in the terminal phosphate. A solvent system has been devised for separation of mono-, di-, tri-, tetra-, penta-, and higher phosphates of thiamine by paper chromatography (55). A method has also been described for separation of TPP from the other phosphates formed from the reaction of thiamine and polyphosphoric acid (56). Fioretti & Mascolo (57) have devised a paper electrophoresis procedure for separation of the phosphoric acid esters of thiamine from the corresponding derivatives of oxythiamine.

Cerletti *et al.* (58) have studied the end products of mild alkaline hydrolysis of the pyrophosphates of thiamine, oxythiamine, riboflavin and adenosine, using paper chromatography and electrophoresis. They find that at room temperature, inorganic phosphate and the organic monophosphates are formed. There is no splitting to a free base and pyrophosphate.

Woolley and De Caro *et al.* (59) have continued their disagreement concerning the effect of pyrithiamine on the thiamine content of brain. Although De Caro's group has shown that the total thiamine (free and bound, measured separately) is decreased in brain to less than one-third normal levels following pyrithiamine administration, Woolley contends that they have not demonstrated a decrease in TPP, since all bound thiamine is not present as TPP. Roux *et al.* (60) suggest that some of the bound thiamine in tissues may exist as polyphosphoric amide esters. Pyrithiamine, but not oxythiamine, inhibits the increase in R.Q. and heat production usually found in the rat following oral administration of glucose (61). This inhibition is independent of the reduction in intestinal absorption of glucose caused by both compounds.

Suzuki & Kishida (62) showed that oxythiamine inhibits growth of *Tetrahymena geleii* W only when added to the medium prior to inoculation of the organism. They suggest that differences in inhibition by pyrithiamine and oxythiamine may be due to the inability of oxythiamine to form a lipothiamide derivative, due to replacement of the NH<sub>2</sub> group attached to



the pyrimidine ring by an OH. Oxythiamine triphosphate competitively inhibits the use of thiamine triphosphate by a yeast carboxylase system (63). Kunz (64) has shown that neopyrithiamine causes characteristic changes in the action current of single nerve fibres during electrotonus, whereas oxythiamine has no such effect.

The pyrimidine moiety of thiamine (pyramin) is a competitive inhibitor of vitamin B<sub>6</sub> for certain heterotrophic microorganisms (65). This appears to be related to the earlier finding (66) that the coenzyme action of pyridoxal phosphate in tyrosine decarboxylation in *Lactobacilli* is inhibited by pyramin phosphate. Conversely, vitamin B<sub>6</sub> compounds overcome the convulsions and death of mice and rats injected with this pyrimidine (also called "toxo-pyrimidine") (67, 68, 69) and competitively inhibit thiamine biosynthesis in *Neurospora* (70). In the presence of a cell-free extract of bakers yeast, pyrimidine phosphate plus thiazole formed thiamine much more rapidly than did the free pyrimidine plus thiazole (71). The phosphates of the pyridoxine compounds appear to be the true competitive substrates in inhibition of thiamine synthesis.

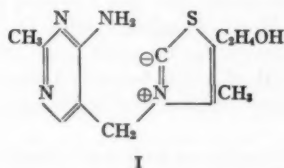
Guthrie *et al.* (72) have shown that several analogues of the pyrimidine moiety of thiamine inhibit growth of amethopterin-resistant mutants of *Bacillus subtilis* and of mutants of *Escherichia coli* (resistant to purine antagonists) more than they inhibit that of the respective parent strains. Amethopterin-induced growth inhibition of *B. subtilis* is effectively blocked by the pyrimidine moiety, thiamine or TPP (73).

Matsukawa & Kawasaki (74) have suggested that as part of the biochemical activity of thiamine, S-acetylcoccarboxylase may acetylate CoA by transacetylation to form acetylCoA. This is based in part on their finding that in the reaction of O,S-diacetylthiamine with a compound having an active thiol group, such as cysteine or glutathione, the S-acyl group is readily transferred to the latter compound. The S-acyl thiamine compounds were as effective as thiamine in preventing thiamine deficiency in rice birds. These workers have also shown that crude S-acetylcoccarboxylase, obtained by acetylating cocarboxylase in an alkaline medium, reacts with cysteine and glutathione, forming N-acetylcysteine and S-acetylglutathione. Kawasaki (75) has prepared crystalline S-acetylthiamine mono- and diphosphates and has confirmed the reaction with glutathione to form S-acetylglutathione. Matsukawa and Kawasaki (74) interpret the polarographic findings of Watanabe & Asahi (76) as evidence that thiamine may exist in the thiol form between pH 7 and 9. Watanabe *et al.* (77) have recently shown that at pH 7, 0.01 per cent of TPP exists in the thiol form. It is suggested (74) that the thiol form of cocarboxylase combines with pyruvate forming a thiolacetal. The product is then dehydrogenated in the presence of DPN, simultaneously losing CO<sub>2</sub>, and the resulting S-acetylcoccarboxylase acetylates CoA to form acetylCoA. Maier & Metzler (78), in a study of the structures of thiamine in basic solution, consider that a free thiol of thiamine may function in a biological system although the average pK value is high. They suggest that

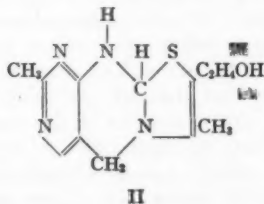


prior reaction with a substrate such as pyruvate at the C-2-position of the thiazolium ring, might decrease the pK value greatly. The data of Watanabe *et al.* (77) show that a small amount of the free thiol of TPP would be available at the pH of biological systems. The demonstration by Yamada *et al.* (79) that the S<sup>35</sup> of labeled methionine is incorporated into thiamine of rat liver both *in vivo* and *in vitro*, suggests that an opening and closing of the thiazole ring may be possible.

Westheimer and co-workers (80, 81) have presented convincing evidence based on reaction in D<sub>2</sub>O, disproving the earlier suggestion of Breslow (82) that the hydrogens of the methylene bridge of thiamine ionize to form an *ylid*, to explain the role of thiamine in catalyzing the decarboxylation of pyruvic acid. Breslow (83) has recently demonstrated clearly that the hydrogen atom in the 2-position of thiazolium salts, including thiamine, ionizes and readily exchanges with the hydrogen atoms of water in neutral solution at room temperature. On this basis, he suggests (84) that the anion formed (I) is involved in the reaction of thiamine and pyruvate in a manner analogous to cyanide catalysis of the benzoin condensation. He postulates that the



carbonyl group adds to the anion at the C-2-position and is decarboxylated readily to form an anion which is stabilized by the adjacent C=N<sup>+</sup> group. Acetaldehyde is then split off. A model condensation product has been synthesized which readily forms benzaldehyde and a thiazolium compound in pyridine solution. Breslow (84) states that previous evidence from model reactions in alkaline solution, which suggest addition of pyruvate to the tertiary thiazole nitrogen of thiamine in the pseudobase form (85), can be explained on the basis of the new mechanism. Maier & Metzler (78) suggest that the NH<sub>2</sub> group of thiamine may add to the thiazolium ring at the C-2-position with simultaneous loss of a proton to form an intermediate tricyclic dihydrothiachromine (II). This conjugation with the electron accepting



centers of the pyrimidine ring may assist in the decarboxylation of the intermediate suggested by Breslow (84). Manzo & Metzler (86) have verified the finding of Mizuhara *et al.* (87) on their model system for the non-enzymatic decarboxylation of pyruvate by thiamine and have confirmed the findings of Breslow (82) of the relative activities of a number of thiamine analogues.

Thiamine pyrophosphate disulfide (TPPDS), which has one-fifth to one-seventh the activity of TPP in the pyruvate oxidation system in liver homogenates of thiamine-deficient rats, has no coenzyme activity in a purified wheat germ apocarboxylase system (88). The liver homogenate reduces the TPPDS to a TPP-active substance before use in pyruvate oxidation. Pyrophosphate inhibition of TPP in the wheat germ apocarboxylase system is competitive, and influenced by the  $Mg^{++}$  level (89). Matsukawa & Iwatsu (90) have devised a new synthesis of dihydrothiamine. This compound has one-tenth to one-fifteenth the activity of thiamine in prevention of avitaminosis in rice birds.

Further modifications (91, 92) of the *L. fermenti* assay for thiamine (93) have been presented and one of these (92) has been applied to the measurement of thiamine in the blood of various species. Deibel *et al.* (94) have proposed the use of *L. viridescens* for thiamine assay and Hayden (95) has devised a colorimetric method using 6-aminothymol, for measuring larger amounts of thiamine. Horie (96) has modified the yeast manometric method for assay of TPP.

Coppock *et al.* (97) have shown no loss of thiamine during the fermentation processes involved in making bread but about a 15 per cent loss during baking. The thermal destruction rates of thiamine in puréed meats and vegetables were studied over a temperature range of 228 to 300°F. and were found to be less than those of the pure vitamin in aqueous or buffered solutions (98). Exposure of raw ground beef to  $\gamma$ -irradiation ( $3 \times 10^6$  rep) destroyed over 60 per cent of the thiamine as measured by biological, microbiological and chemical methods (28). Extensive destruction of thiamine has also been reported in irradiation of turkey meat (99). Decomposition products of thiamine solutions subjected to  $\gamma$ -irradiation were studied by Groninger & Tappel (100).

Chikubu and co-workers (101, 102, 103) have examined the distribution of thiamine in wheat, barley, rice and other cereals at different stages of maturity by photographing the thiochrome fluorescence observed in slices of the tissues. By use of somewhat similar histochemical techniques with rat tissues, the absorption of thiamine was found to be much more marked in the small intestine than in the stomach or large intestine (104). Banerjee & Guha (105) have shown that although the thiamine contents of various strains of cereals differ widely, samples of the same strains taken in two successive years show nearly the same thiamine content, indicating that in most cases the thiamine value is a stable characteristic of the variety.

The thiamine content of breast milk of poor human subjects in India

averaged 12.2 mcg. per 100 ml. (range 3 to 25 mcg.), and was in general lower than that usually found in American and English subjects (106).

#### LIPIC ACID (THIOCTIC ACID)

In the papers discussed in the following review, the trivial name lipoic acid is used to designate  $\alpha$ -lipoic acid (6:8-thioctic acid). The chemistry and function of lipoic acid have recently been reviewed by Reed (107) and Grisebach (108).

A new synthesis of DL-lipoic acid was reported by Segre *et al.* (109), who oxidized 2-acetoxyethylcyclohexanone to the 1,6-lactone of 6-hydroxy-8-acetoxyoctanoic acid. Reaction of the lactone with thiourea and a strong mineral acid, followed by alkaline hydrolysis of the isothiuronium salt and oxidation of the dithiol acid thus formed, yielded DL-lipoic acid in 19 per cent over-all yield from cyclohexanone. Bullock *et al.* (110), described a modification of their previously published (111) method of synthesis of lipoic acid, in which the condensation of methyl or ethyl chloroformylvalerate with ethylene was carried out in ethylene chloride, and the intermediate  $\beta$ -chloroketone dehydrohalogenated with sodium acetate to yield the corresponding 6-oxo-7-octenoate, which was then converted to lipoic acid by known procedures. Use of propylene rather than ethylene in the above reaction sequence, with formation of the corresponding nine-carbon intermediates, yielded 8-methyl-lipoic acid, which was obtained as a molecular compound containing both pairs of diastereoisomers. In a second paper, Bullock *et al.* (112) described a method for the production of lipoic acid in relatively large amounts by an economical and rapid procedure. Readily available intermediates such as ethyl 6-oxo-7-octenoate or the 8-acetylthio or benzylthio derivatives were reduced by excess hydrogen sulfide and hydrogen in the presence of cobalt polysulfide, a sulfactive catalyst. Preparation of lipoic acid in much lower yield by direct reduction of a carbonyl group was described previously by Soper *et al.* (113).

Wladislaw (114) reported synthesis of 3,5-dithiopentanoic acid, a lower homologue of lipoic acid. Previously, Claeson (115) had described the synthesis of the 4-carbon homologue of lipoic acid by reaction of  $\alpha$ ,  $\gamma$ -dibromobutyrate and potassium thiolactate, followed by oxidation of the resulting dimercaptobutyrate to racemic 1,2-dithiolane-3-carboxylic acid. Examination of the biological activity of these homologues would be of interest.

Brockman & Fabio (116) synthesized 6-ethyl-8-mercaptooctanoic acid and several of its homologues, and Broquist & Stiffey (117) found that this lipoic acid analogue inhibited the growth of the yeast *Torula cremoris*. The inhibition was not reversed by lipoic acid over a wide range of concentration but was reversed competitively by a number of fatty acids, including acetic acid, oleic acid and palmitic acid. It was also reversed noncompetitively by substrate amounts of both isoleucine and valine. The structural analogy between 6-ethyl-8-mercaptooctanoic acid and 6-acetylthio-8-mercaptooctanoic acid, known to be a biologically active form of lipoic acid (118) suggested to

Albrecht (119) that the former might interfere with acyl transfer. 6-Ethyl-8-mercaptooctanoic acid did not inhibit the anaerobic decarboxylation of pyruvate by extracts of *E. coli*, but inhibited pyruvate oxidation, thus demonstrating that 6-ethyl-8-mercaptooctanoic acid interferes beyond the TPP stage. When the enzymically mediated reaction "acetyl phosphate + CoASH  $\rightarrow$  S-acetylCoA + iP" was coupled with the arsenolysis reaction, it was found that 6-ethyl-8-mercaptooctanoic acid markedly interfered with phosphotransacetylase activity. This effect was reversed by dihydrolipoic acid, suggesting the possible involvement of the latter in transacetylase reactions.

Syntheses of DL-dimethyl, DL-dibenzyl, DL-8-S-methyl-6-S-benzyl and DL-8-S-benzyl-6-S-methyldihydrolipoic acids were described by Nakano & Sano (120) who reported that all of these reduced lipoic acid analogues were obtained as oils except the DL-dibenzyl compound, which was crystalline. Synthesis of DL-dimethyldihydrolipoic acid was reported previously by Wagner *et al.* (121), who found no lipoic acid activity of this compound by the enzymatic pyruvate oxidation factor assay of Gunsalus *et al.* (122). Nakano & Nagasaki (123), however, prepared DL-dimethyldihydrolipoic acid which possessed weak lipoic acid activity in the same assay, suggesting that their preparation may not have been chemically pure. Nakano (124) reported that acetylation of ethyl DL-dihydrolipoate with acetic anhydride in pyridine at temperatures below 15°C. yielded mostly ethyl DL-8-S-acetyldihydrolipoate, although some ethyl DL-6-S-8-S-diacyldihydrolipoate was also formed. This is not in agreement with the results of Gunsalus *et al.* (118), who found that the secondary -SH group of dihydrolipoic acid was not acetylated by chemical reagents under similar conditions. Nakano (125) studied *in vitro* transacetylation between ethyl DL-diacyldihydrolipoate and aniline or benzylamine. With aniline, only the 8-S-acetyl group was transferred to form acetanilide, whereas with benzylamine, both acetyl groups were transferred, with the formation of benzylacetamide.

Reed *et al.* (126) have continued their studies on a lipoic acid-activating system involved in dismutation of pyruvate and  $\alpha$ -ketobutyrate in certain microorganisms. A purified pyruvate oxidation system from *E. coli*, which contained firmly bound lipoic acid, was inactivated by treatment with an enzyme fraction from *Streptococcus faecalis* which catalyzes the hydrolysis of amides and esters of lipoic acid. Pyruvate oxidation activity was restored by incubating the inactive preparation simultaneously with lipoic acid and ATP (or synthetic lipoyl adenylate) and the lipoic acid-activating enzyme system from *E. coli*.

Sanadi & Searls (127) found that mammalian  $\alpha$ -ketoglutaric dehydrogenase, an enzyme complex which contains lipoic acid or a microbiologically active derivative (128, 129), catalyzed the reversible oxidation of reduced DPN by lipoic acid or lipoamide. The (+) isomer of lipoic acid was active in the reaction while the (-) isomer was inactive, although the presence of the inactive isomer had no effect on the velocity of reaction. The pH optima for  $\alpha$ -ketoglutarate oxidation and lipoamide reduction were between 7.0

and 7.4, whereas with lipoate as substrate the activity was greater at pH 6 than at pH 7. The concentration needed for half-maximal velocity with DL-lipoamide was much less than with DL-lipoate. These data on pH optima and  $K_m$  suggested to Sanadi & Searls (127) that lipoamide may be more closely related to the naturally bound cofactor than is lipoic acid.

Drummond & Stern (130) have studied some enzymatic reactions of 6,8-dithiioctanoic acid (dihydrolipoic acid) and its acetoacetic thioester. They observed that acetoacetate synthesis by partly purified ox and chicken liver enzyme preparations from acetylCoA (generated by phosphotransacetylase from CoASH and acetyl phosphate) required the addition of a mono- or dithiol compound. Among active thiols, DL-dihydrolipoic acid had the greatest activity, half maximum activation occurring with  $1 \times 10^{-4}$  M DL-dihydrolipoic acid, as compared with  $3.5 \times 10^{-4}$  M dimercaptopropanol and  $2 \times 10^{-3}$  M glutathione. Formation of acetoacetate and DL-dihydrolipoate from synthetic S-acetoacetyl-DL-dihydrolipoic acid [presumably the 8-ester in view of the results of Gunsalus *et al.* (118)] was catalyzed by enzyme fractions contained in liver but apparently not in other tissues. The enzyme was purified fortyfold from chicken liver and found to differ from acetoacetyl-S-glutathione thioesterase. The enzyme fractions in liver also catalyzed the reaction "DL-acetoacetyl-S-pantetheine (or CoA) + DL-dihydrolipoic acid  $\rightarrow$  acetyl-S-pantetheine (or CoA) + DL-S-acetyldihydrolipoic acid." It is not known whether the 6- or 8-S-acetyldihydrolipoate was formed. In any case, this enzymatic reaction represents a novel synthesis of S-acetyldihydrolipoate, in that it involves transacetylation with a 4-carbon fragment and utilizes both isomers of dihydrolipoate, whereas the synthesis of (+) 6-S-acetyldihydrolipoate, reported by Gunsalus *et al.* (118), involved reaction of acetyl-CoA and (-) dihydrolipoate, under the influence of dihydrolipoate transacetylase.

Machlis (131) found the growth of the watermold (*Allomyces macrogynus*) in agitated liquid culture was reduced approximately fifty per cent in the presence of  $5 \times 10^{-8}$  M lipoic acid. The specific effect on growth was to increase the lag phase, although growing mycelia were also somewhat inhibited. Lipoic acid inhibition was prevented by acetate, pyruvate, aspartate, glutamate, arginine and proline. The analogue 8-methylipoic acid was also inhibitory, but 5,8-dithiioctanoic acid, glutathione, thioglycolate, cystine and cysteine had no effect. Machlis (131) postulates that an analogue or other form of lipoic acid is necessary for growth of this organism.

Grisebach *et al.* (132) prepared  $S^{35}$ -labeled lipoic acid by the method of Adams (133) and studied its metabolism in algae. Evidence was obtained that the major metabolic products of lipoic acid in *Scenedesmus* or *Chlorella* are lipoic acid esters of glycerol or a normal alcohol, or the corresponding sulfoxides, and possibly water-soluble lipoic acid-protein complexes, all located in the chloroplasts. Incubation of *Scenedesmus* with  $S^{35}$ -labeled lipoic acid under aerobic and anaerobic conditions showed that aerobic metabolism favors production of the ester form. Elucidation of its possible

role in photosynthetic processes will be awaited with interest. In additional experiments, Grisebach *et al.* (132) reported failure of attempts to prepare lipothiamide (the amide of lipoic acid with thiamine) by the method of Reed & DeBusk (134) or by reaction of lipoic acid and thiamine in the presence of dicyclohexylcarbodiimide.

Lipoic acid influences the development of the eggs of the sea urchin (*Paracentrotus lividus*), producing an animalizing effect according to Runnström (135). Further evidence was presented that lipoic acid has no effect on the growth of chicks fed nutritionally complete or deficient diets (136).

There have been several reports that lipoic acid may be of some value in treatment of certain cases of hepatic insufficiency in man. Kuhn (137) administered 8 to 25 mg. of lipoic acid intravenously to patients with liver disease and noted a fall in the abnormally high blood pyruvate and  $\alpha$ -ketoglutarate concentrations found in this disorder. Previously, Rausch (138) had treated twenty-two cases of liver disease with lipoic acid, and concluded, as did Steigmann & Canahuati (139) that although striking clinical improvement was observed in only some of the cases, the results obtained warranted further experimentation. Thompson *et al.* (140) reported that administration of lipoic acid lowered the elevated lactic and pyruvic acid concentrations in the blood of patients in hepatic coma. However, in apparently better controlled experiments, Summerskill *et al.* (141) found that lipoic acid had no consistent effects on arterial blood ammonia, pyruvate or  $\alpha$ -ketoglutarate levels in patients with hepatic coma. Similarly, Dawson *et al.* (142) also found no constant effects of administration of lipoic acid on the clinical state or blood keto-acid concentration of patients in hepatic precoma. Obviously further experimentation is necessary before the possible role of lipoic acid in the therapy of liver disease can be fully elucidated.

#### PANTOTHENIC ACID

The pantothenic acid content of a variety of materials, including grape musts (143), cheeses (144) and royal jelly (145) has been reported. The values for pantothenic acid in a large number of foods, obtained by Zook *et al.* (146), may supersede some earlier values, since bound forms of the vitamin were released by use of the double enzyme system of Novelli *et al.* (147), prior to assay with *Lactobacillus plantarum*. Improvements in the *L. casei* assay for pantothenic acid have been reported (148). A method has been described (149, 150) for determination of the CoA content of tissues, based on enzymatic liberation of pantetheine by intestinal phosphatase, followed by measurement of the pantetheine microbiologically. The pantetheine can be determined in the presence of free pantothenate, under conditions in which each of the two factors is devoid of any stimulatory activity towards the other.

Barboriak *et al.* (151) concluded that the pantothenic acid requirement of the growing rat lies between 0.8 and 1.0 mg. per 100 gm. of diet, which is in agreement with previous findings (152). These authors also found that the



same level of pantothenate was necessary for optimal acetylation of injected sulfonamide in adult rats. The low pantothenic acid requirement for maximum growth in rats reported by Blunt *et al.* (153) is probably invalid, since it appears from the poor growth obtained that the purified diet used was not adequate. The pantothenate requirement of the growing pig has been studied. As has been elsewhere stated (154), the failure of Barnhart *et al.* (155) to obtain pantothenic acid deficiency in any of their groups of pigs may be related to the ascorbic acid content of the basal diet used, and/or to the fact that the basal diet was not pantothenate-free. McKigney *et al.* (156) concluded that 6.7 mg. pantothenic acid per kg. diet was sufficient for normal weight gain in weanling pigs, but that this did not prevent the appearance of pantothenic acid-deficiency symptoms such as diarrhea and inflammation of the small intestine. Chlortetracycline appeared to spare the dietary requirement for pantothenic acid in the pig.

In carefully conducted studies on the role of pantothenic acid in embryonic development, Nelson *et al.* (157) observed multiple congenital abnormalities in rat fetuses when a pantothenic acid-free diet was given to the mother either four to ten days prior to breeding or on the first day of gestation, and continued throughout pregnancy. The abnormalities included cerebral and ocular defects, digital hemorrhages and edema, cardiovascular anomalies, cleft plate, hydronephrosis and hydroureter. Fetal development was only slightly disturbed when the vitamin deficiency was restricted to the first twelve to fourteen days of pregnancy. However, addition of the antimetabolite  $\omega$ -methyl-pantothenic acid to the pantothenate-free diet for the last two or three days of this period accentuated the deficiency.

Pantothenic acid deficiency, characterized by dermatitis, excessive nasal secretion, loss of appetite, reduced growth rate and eventual death, was produced in young calves fed a synthetic diet devoid of the vitamin (158). Muscle edema and changes in nervous tissue were also noted. Gantt (159) found impaired conditioned reflexes in dogs fed a pantothenic acid-deficient diet prior to observable behavioral, neurologic, or hematologic changes. A return to an adequate diet restored the ability of the animals to differentiate various auditory stimuli. Goetinck *et al.* (160) found that injection of  $\omega$ -methyl-pantethine into fertile turkey eggs before incubation resulted in greatly increased embryonic mortality. Embryos surviving to the final week of incubation exhibited micromelia and leg malformations similar to those previously found in pantothenic acid-deficient turkey embryos by Kratzer *et al.* (161).

An inhibitory effect of  $\omega$ -methyl-pantethine on sulfanilamide acetylation in pigeon liver homogenates was observed at concentrations which were ineffective against citrate formation (162). Bis-( $\beta$ -pantoylaminoethyl)-disulfide inhibited citrate formation at concentrations ineffective against sulfanilamide acetylation. Higher concentrations of either compound inhibited both systems.

Relationships between pantothenic acid and the adrenal glands have



been studied by several investigators (163 to 166). Eisenstein (165) has reviewed the effects of dietary factors, including pantothenate, on production of adrenocortical hormones. The ability of the isolated adrenal cortex of rats to secrete steroid hormones under the influence of ACTH was measured using spectrophotometric methods (166). The results showed that secretion of adrenocortical hormones is decreased in the pantothenic acid-deficient rat as compared to pair-fed or *ad libitum* fed control animals.

Barboriak & Krehl (167) have investigated the influence of pantothenic acid and growth hormone on the growth process in young hypophysectomized rats. Unlike intact pantothenate-deficient animals, in which growth hormone apparently does not promote further growth (168), hypophysectomized rats fed a pantothenate-deficient diet responded markedly to growth hormone. Hemorrhages and congestion were frequently observed in the adrenals of pantothenic acid-deficient hypophysectomized animals which had received growth hormone, indicating that integrity of the adrenal-pituitary axis is not necessary for the development of adrenal lesions.

Gounelle & Richet (169) observed that the levels of free pantothenic acid in blood and urine of normal human subjects, were markedly elevated within four hours after administration of a test dose of pantothenol. Ueshima *et al.* (170) reported that urinary excretion of pantothenic acid was reduced in patients with liver disease as compared to normal controls. Some correlation was found between urinary pantothenate and those liver function tests in which pantothenic acid is known to be involved. Wenneker (171) found that following partial hepatectomy in the rat, the regenerating liver shows a reduced concentration of CoA, which is in agreement with the low pantothenate levels found by Ferrari (172). Previously, Severi & Fonnesu (173) had found a similar reduction of hepatic pantothenate concentration in rats with fatty livers induced by  $\text{CCl}_4$ .

There have been several reports (174 to 177) on the use of relatively large amounts of pantothenate in treatment of clinical syndromes. On the basis of results obtained with over 100 patients, Luisi (174) concluded that calcium pantothenate, administered orally or parenterally in daily doses of 500 mg., is an effective therapeutic agent for treatment of cramps in pregnant women. Several authors have reported favorable results from the use of pantothenic acid or derivatives in treatment of postoperative atony of the bowel. Luraschi (175) concluded that calcium pantothenate, injected intramuscularly for four to five days at a daily dose of 500 mg., had such favorable effects on bowel atony following Caesarean section or adnexectomy as to make it the treatment of choice. Schulte (176) reported studies on the use of pantothenol in 82 unselected cases of laparotomy for various diseases. The patients received 500 mg. pantothenol or a placebo immediately following operation and every six hours thereafter. Pantothenol significantly shortened the time of postoperative bowel atony as measured by the time required for the appearance of bowel sounds, the passage of flatus and the

passage of the first stool. Galeotto (177) concluded from preliminary clinical studies that pantothenate has value as an anti-curare agent.

Manthei (178) found that guinea pigs injected with toxic doses of isoniazid exhibited a transient peripheral neuropathy characterized by initial hyperreflexia, hyperesthesia, and extensive muscle flaccidity. Isoniazid toxicity was prevented by administration of calcium pantothenate, but not by pyridoxal hydrochloride, administered eighteen hours before the challenging dose of isoniazid. In the mouse, however, concomitant administration of calcium pantothenate gave no protection against isoniazid, whereas pyridoxine was most effective (179).

The toxicity of pantetheine has been studied by Knott *et al.* (180), who found that the LD<sub>50</sub> of pantetheine administered intraperitoneally in mice was between 5 and 7.5 gm. per kg. body weight. This is much higher than the LD<sub>50</sub> for pantothenic acid given intraperitoneally in the mouse, which is approximately 0.92 gm. per kg. body weight (181).

Garattini *et al.* (182, 183) synthesized several derivatives of phenylacetic acid and found them to inhibit CoA, as evidenced by decreased acetylation of sulfanilamide and of acetylcholine synthesis *in vitro*. The most effective compound tested was  $\alpha$ -*p*-biphenylbutyric acid which was also effective *in vivo*. Because of the previously demonstrated importance of CoA for cholesterol biosynthesis (184, 185), Garattini *et al.* (182, 183) studied the effects of CoA-inhibitory drugs on cholesterol metabolism. Hypercholesterolemia and hyperlipemia were produced in rats by administration of Triton, a polymer of *p*-isooctylpolyoxyethylenephenol.  $\alpha$ -*p*-Biphenylbutyric acid, administered intravenously in dosage of 200 mg. per kg. body weight exhibited marked anti-hypercholesterolemic and anti-hyperlipemic activity. Several investigators have noted interrelationships between pantothenic acid and other vitamins. Okuda (186) found that the liver vitamin B<sub>12</sub> concentration was increased in pantothenic acid-deficient rats, in agreement with the findings of Radhakrishnamurty & Sarma (187). Sundaram & Sarma (188) studied nicotinic acid metabolism in normal and pantothenate-deficient rats, with special reference to the excretion of nicotinuric acid, and found that a much lower proportion of administered nicotinic acid was excreted as this conjugated form, and a much higher proportion as the free form, in pantothenic acid-deficient rats. These results suggest that CoA is necessary for the conjugation of nicotinic acid and glycine to form nicotinuric acid, in a manner analogous to its role in the biosynthesis of hippuric acid from benzoic acid and glycine. From urinary excretion studies in control and pantothenate-deficient rats, Thangamani & Sarma (189) concluded that pantothenic acid is required for the conversion of glucose cyclo-acetoacetate to ascorbic acid. Rogers & Campbell (190) have observed that at suboptimal levels of pantothenic acid, the growth of *L. leichmannii* 313 was stimulated by riboflavin.

Several metabolic reactions in which CoA is known to be involved, in-

cluding acetylation of aromatic amines, citrate synthesis and fatty acid synthesis, have been reviewed by Pütter (191), who interprets them on the basis of polar mechanisms. Involvement of pantothenic acid in the biosynthesis of heme is indicated by the results of Schulman & Richert (192). Whole blood from pantothenic acid-deficient ducklings was found to incorporate less glycine-2-C<sup>14</sup> or succinate-2-C<sup>14</sup> into heme than that from normal animals. In severe pantothenic acid deficiency, there was moderate diminution in the incorporation of  $\delta$ -aminolevulinic acid-2,3-C<sup>14</sup> into heme. Injection of pantothenate into the deficient ducklings one hour before removal of blood restored glycine incorporation into heme to normal. The addition of calcium pantothenate *in vitro* did not restore synthesis to normal. Biosynthesis of hematin compounds in a hemin-requiring strain of *Micrococcus pyogenes* has been studied by Jensen (193), who found that CoA is needed for the synthesis of catalase, presumably in the transfer of hemin to the apocatalase.

Synthesis of pantothenic acid and its congeners by wild type *Neurospora crassa* and by a pantothenicless mutant has been studied by Ikawa & O'Barr (194) using bioautographic techniques. After four days incubation, culture filtrates of wild type *Neurospora* contained principally CoA, but on longer incubation, the CoA content of the filtrate decreased and the pantothenic acid and pantetheine levels increased. Little or no pantoic acid was found. Culture filtrates of the pantothenicless mutant, grown with minimal pantothenate for four days, contained substantial amounts of pantoic acid, but little or no pantothenic acid or CoA. Small amounts of CoA were detected, however, if the culture was grown with large amounts of pantothenic acid. CoA and other pantothenic acid congeners did not support growth of the mutant. The results suggest that the enzyme system for coupling  $\beta$ -alanine and pantoic acid is either lacking in the mutant or is inhibited by a factor in the culture medium.

McIntosh *et al.* (195) have purified an enzyme system from *E. coli* which catalyzes the condensation of  $\alpha$ -ketoisovalerate and formaldehyde to yield ketopantoic acid ( $\alpha$ -keto- $\beta,\beta$ -dimethyl- $\gamma$ -hydroxymethylbutyric acid). The ketopantoic acid was shown to have pantothenate activity for *E. coli* mutant W-3. Synthesis of a large number of S-acyl pantetheines has been reported by Felder & Pitré (196). The reactions involved exchange of S-acyl mercaptoethylamine with a mixed anhydride of pantothenic acid to yield the resulting S-acyl pantetheine in fifty to seventy per cent yield. That CoA is not necessary for the enzymatic acylation of pantetheine in cell-free extracts of *L. helveticus* 80 is indicated by the results obtained by Brown (197). Pantetheine was acetylated more effectively by acetyl phosphate than by acetylCoA. The utilization of pantothenate and pantothenylcystine for growth of a mutant of *L. helveticus* 80, and the conversion of pantothenylcystine to pantetheine by resting cell suspensions of this mutant was reported (198). The parental culture failed to use pantothenate and pantothenylcystine for growth, but utilized pantetheine for this purpose, possibly because of inability to absorb pantothenate and pantothenylcystine from the culture medium.

A new resolution of pantolactone for synthesis of calcium pantothenate has been reported by Kagan *et al.* (199). Reaction of pantolactone with D-galactamine yielded a pair of diastereoisomeric N-D-dulcetyl-2,4-dihydroxy-3,3-dimethylbutyramides, which were separated by fractional crystallization. Acid hydrolysis of the (+) diastereoisomer gave D(-)pantolactone and D(-)galactamine in 94 and 90 per cent yields, respectively. Coupling of the calcium salt of  $\beta$ -alanine with D(-) pantolactone in methyl cellosolve yielded crystalline D(+)calcium pantothenate in 94 per cent yield based on pantolactone.

#### RIBOFLAVIN

Beinert (200, 201) has continued his studies (202) on the production of semiquinoid intermediates during the reduction of flavins. By means of special techniques for recording rapid spectral changes, spectra were obtained during reduction with substrate or dithionite of several flavoenzymes, including old yellow enzyme from yeast, L-amino acid oxidase from snake venom and acyl dehydrogenase ( $C_4-C_{16}$ ) from pig liver. The spectral evidence indicated the appearance of a transient intermediate, characterized by a broad absorption band in the region of 500 to 650 m $\mu$ , during oxidation-reduction of these enzymes. This absorption band is typical for the semiquinone of free flavin at neutral pH (202) and could be ascribed to an intermediate oxidation state of the prosthetic flavin of the enzymes rather than to an enzyme-substrate complex, since it was also observed during reduction of the acyl dehydrogenases in the absence of substrate. However, strong enzyme-substrate complexes are formed, as indicated by data obtained in the presence and absence of substrate, which show that at all oxidation levels, addition of substrate renders the prosthetic flavins of these enzymes inaccessible to further reduction or reoxidation. As a result the oxidation state is quasi-frozen after addition of substrate.

Ehrenberg (203) demonstrated by paramagnetic resonance absorption that the semiquinone of FMN was present in aqueous solution, the milieu of enzymatic reactions *in vivo*. Ehrenberg used frozen samples, which diminished the dielectric losses greatly, and worked at the temperature of liquid nitrogen, which improved the sensitivity and "locked" the reaction at a desired point. Polarographic evidence for the formation of semiquinones of FMN and FAD has been presented (204). The complete polarographic waves of FMN and FAD consist of a normal reduction wave and an "anomalous" adsorption wave occurring at a more negative potential (204, 205).

In studies on the reaction between riboflavin and phenols in water solution, Yagi & Matsuoka (206) found that phenol destroyed the characteristic fluorescence of riboflavin, mainly through complexing with the isoalloxazine ring of the vitamin.

The products formed by the action of light on riboflavin were studied by several investigators. Shimizu (207) found that in neutral solution the products of photolysis, calculated on the basis of 1 mole of riboflavin, were

as follows: 0.82 mole of lumichrome, 0.74 mole of formaldehyde, 0.09 mole of formic acid and about 1 mole of an unidentified sugar. No lumiflavin was observed. In alkaline medium, 0.65 mole of lumiflavin, 0.09 mole of lumichrome, 0.10 mole of formaldehyde and 0.06 mole of formic acid were detected per mole of riboflavin. In addition, four-carbon compounds, possibly mixtures of erythrose and erythronic acid, were produced. Fukamachi & Sakurai (208) irradiated riboflavin in alkaline solution containing hydrogen peroxide and isolated 6,7-dimethyl-flavin-9-acetic acid, as well as lumiflavin and lumichrome. Starting from lumiflavin and its 2-thio analogue, Hemmerich & Erlenmeyer (209) prepared a series of acyl-leuko compounds, as well as several substituted lumiflavins.

Several investigators have studied modifications of media for the industrial production of riboflavin by the microorganism *Eremothecium ashbyii* (210, 211, 212). Stárka (213) found that synthesis of riboflavin by *E. ashbyii* occurred after cessation of growth, with the maximum production occurring in the fourth or fifth day of cultivation of the organism in shaken culture on glucose-peptone-yeast autolysate medium. Although adenine is known to be a direct precursor of riboflavin in *E. ashbyii* (214), it has no influence on the synthesis of the vitamin in the related organism *Ashbya gossypii* (215). An increase in riboflavin synthesis by *A. gossypii* resulted from addition to the medium of various surface active agents such as fatty acid esters of sorbitan and polyoxyethylene. Mitra (216) studied sources of carbon and nitrogen for a riboflavin-producing mutant yeast subsequently identified (217) as a new species tentatively named *Candida ghoshii*. Maximum riboflavin production was observed with glucose, fructose or sucrose as sole source of carbon. The yeast was unable to utilize lactose. Asparagine was the most effective nitrogen source tested. Bulmash & Weaver (218) have found riboflavin to be required by three strains of *Clostridium histolyticum* grown on synthetic medium.

The stimulatory effect of a vegetable diet on synthesis of riboflavin in man reported by Iinuma (219) may be due to the cellulose contained in the vegetables, in view of the results of Nagase & Fujita (220) who noted that addition of cellulose to a meat diet resulted in increased fecal excretion of riboflavin in a human subject.

Two new types of riboflavin-decomposing bacteria, both motile nonspore forming Gram-positive rods, were isolated from human feces by Hamada *et al.* (221). Both organisms decomposed riboflavin to lumichrome. It is of interest that the organisms were not detected in the feces of breast-fed infants, but were most frequently observed in feces of elderly subjects and older children. Whether organisms of this type play an important role clinically, as has been shown with thiamine-decomposing bacteria (1), is not known.

An inhibitory effect of iron salts on riboflavin production by a strain of *Clostridium acetobutylicum* has been reported (222). In studies on trace metal requirements and enzyme systems in a riboflavin-requiring mutant of *Neurospora crassa*, Nicholas (223) observed that the activity of iron-con-

taining enzymes was markedly increased in riboflavin deficiency, but that of several flavoenzymes was decreased. Addition of boiled pig heart extract, a source of FAD, to extracts of riboflavin-deficient mycelia restored the activity of flavoenzymes such as DPNH oxidase, TPNH oxidase and nitrite reductase almost to normal, but did not influence the activity of nitrate reductase, a molybdoflavoenzyme. Evidence was obtained for alternative pathways of electron transfer in the fungus. When riboflavin is deficient, oxygen is probably the main terminal electron acceptor. When riboflavin concentration is optimal, however, nitrate acts as the terminal acceptor of electrons. Thus, the electron transfer mechanism of this organism involves an iron system when riboflavin is deficient, and a molybdoflavoprotein system when adequate amounts of riboflavin are present. Iron deficiency was readily produced in the mutant when riboflavin was deficient because of the increased need for iron enzymes. The molybdenum requirement was decreased under conditions of riboflavin deficiency because of decreased production of molybdoflavoproteins. The role of metals in the flavin containing enzymes has been reviewed (224).

By utilizing differences in the fluorescence spectra, Ohnesorge & Rogers (225) developed a fluorometric method for determination of riboflavin and thiamine in mixtures. Schaus & Kirk (226) have studied the riboflavin concentration in brain, heart and skeletal muscles of human subjects from seven days to ninety-two years of age. No effect of age on tissue riboflavin concentration was observed.

In an extensive study, Horwitt *et al.* (227) found that a diet low in niacin and tryptophan had no influence on the development of ariboflavinosis in man. Masuda *et al.* (228) have reported clinical observations of so-called "Shibi-Gatchaki," an endemic disorder found among the inhabitants of the northernmost province of the main island of Japan. The syndrome is characterized by lassitude, severe anal and genital itching, glossitis, dermatitis and ocular lesions. Riboflavin excretion is very low and that of N-methylnicotinamide somewhat lower than normal. Thiamine excretion, however, is only slightly depressed. The authors concluded that the condition is of nutritional origin and appears to be mainly a hyporiboflavinosis, with some admixture of pellagra. Treatment with a mixture of thiamine, riboflavin, niacin, folic acid and vitamin B<sub>12</sub> caused rapid regression of the symptoms in most patients. Variation in riboflavin excretion in man has been studied by Hegsted *et al.* (229), who noted that the variation in riboflavin excretion per hour and per gram of creatinine was of the same order of magnitude, while the excretion per ml. of urine was more variable in most subjects.

Gershoff & Hegsted (230), found that riboflavin deficiency symptoms in the cat were accentuated on high fat diets. Diminished intestinal synthesis of riboflavin on these diets may account for these findings. Witting *et al.* (231) reported that riboflavin protected growing rats against the toxic effects of autooxidized fats, but has no influence on the toxicity of thermally polymerized fats. Nelson *et al.* (232) observed impaired carbohydrate utiliza-



tion in riboflavin-deficient rats, although carbohydrate absorption was normal. Changes in factors related to resistance to infection appear to be approximately the same in riboflavin deficiency as in inanition (233).

Interrelationships between riboflavin and other vitamins have been reported. A favorable effect of ascorbic acid on the growth of rats fed a riboflavin-deficient diet, similar to that observed previously by Daft & Schwarz (234), was reported by Terroine (235). It is of interest that although ascorbic acid stimulated weight gain, it had no influence on the hepatic riboflavin concentration. Rogers & Campbell (190) found that growth of *L. leichmannii* 313 was stimulated by riboflavin at suboptimal levels of pantothenic acid. A lower concentration of tissue nicotinic acid has been reported in rabbits deficient in both nicotinic acid and riboflavin as compared with riboflavin deficiency alone (236). Rats deficient in riboflavin had significantly lower concentrations of vitamin B<sub>12</sub> in liver, kidney, and heart (237).

The effects of ionizing radiations on the riboflavin content of foods have been studied. Day *et al.* (238) observed that  $\gamma$ -irradiation of ground raw beef (approximately  $3.0 \times 10^6$  rep) resulted in the destruction of about 10 per cent of the vitamin. Ziporin *et al.* (239) found practically no destruction of riboflavin in several foods, except in turkey meat, in which approximately 50 per cent of the vitamin was destroyed by exposure to  $6.0 \times 10^6$  rep of  $\gamma$ -irradiation. Thomas & Calloway (99) have reported only a 17 per cent loss of riboflavin in turkey meat under similar conditions.

Kuwada *et al.* (240, 241) described the preparation of FAD of 81 per cent purity from cultures of *E. ashbyii*. FAD was isolated by column partition-chromatography on starch. The crude material was purified by reduction with sodium hydrosulfite and subsequent recovery by aeration. In studies on FAD biosynthesis using P<sup>32</sup>-labeled FMN, Hotta *et al.* (242) demonstrated that the phosphate of FMN was incorporated *in toto* into FAD. Manson & Modi (243) have studied the occurrence and metabolism of FAD in milk of the lactating sow and cow. Free FAD was absent in ultrafiltrates of cows' milk, but was present in sows' milk to the extent of 0.15 to 0.2  $\mu$ g. per ml. In confirmation of previous studies (244), however, sows' milk was found to contain no FMN or free riboflavin. All of the FAD in cows' milk, but only part of that in sows' milk was bound to protein, from which it could be freed by heating. Bovine milk, unlike sows' milk, contains a system of enzymes capable of converting FAD to riboflavin. No xanthine oxidase was found in sows' milk, although at least part of the combined FAD in cows' milk was found to be present as xanthine oxidase.

The flavin nucleotides and flavoproteins have been reviewed by Huennekens (245). Hemoglobin reductase from human erythrocytes and DPNH peroxidase from *S. faecalis* have recently been shown to be flavoenzymes (246, 247). Singer *et al.* (248) isolated succinic dehydrogenase from beef liver mitochondria in a state approaching homogeneity as measured by physico-chemical criteria. They found it to be a ferroflavoprotein containing 4 atoms of ferrous (non-hemin) iron and a mole of flavin per mole of protein. Eichel



(249) obtained evidence from atabrine inhibition studies that flavin is involved in the over-all succinoxidase system of *T. pyriformis* S, but not in the primary dehydrogenase reaction when phenazine methosulfate is used as an electron acceptor. Tong *et al.* (250) found that addition of FMN markedly increased conversion of carrier iodide to iodoprotein by mitochondrial-microsomal fractions isolated from sheep thyroid glands. Evidence was obtained that the stimulatory action of the flavin is enzymatic in nature and is inhibited by thiouracil, thiocyanate, cyanide and certain aromatic goitrogens, but not by catalase, malonate, perchlorate, antimycin A, or monofluoroacetate.

Several workers have been interested in bioluminescence. Nitrate reductase from *Achromobacter fischeri* (a luminescent salt water bacterium) was purified and separated from other electron-transport systems by Sadana & McElroy (251). The enzyme catalyzes the reduction of nitrate with reduced benzyl viologen as an electron donor. DPNH serves as an electron donor only if a bacterial DPNH-cytochrome reductase is added, in which case flavin is required in the system. Reduced FMN can also serve as an electron donor. Cormier & Totter (252) reported that FMN is not destroyed during luminescence of *A. fischeri* although dodecyl aldehyde disappears during the reaction. Reduced FMN and the enzyme luciferase were required for the disappearance of the aldehyde in the luminescence reaction. Quantum efficiency data supported a previous proposal by McElroy & Green (253) that peroxidation of long-chain aldehydes furnishes the necessary energy for excitation of FMN which subsequently leads to light production. Methemoglobin, which inhibits the luminescence of *A. fischeri*, is reduced by cell-free extracts of *A. fischeri* and *E. coli* (254). This reduction is stimulated by addition of flavin. An enzyme from *E. coli* capable of reducing methemoglobin was partially purified and shown to possess the properties of a diaphorase. This enzyme system reduced methemoglobin in the presence of DPNH or TPNH, methylene blue and FMN. FAD and riboflavin were less active than FMN. Under anaerobic conditions, methemoglobin was quantitatively reduced by reduced FMN. The authors concluded that any flavoprotein with the properties of a diaphorase, or a readily dissociable flavin moiety, can serve as a "methemoglobin reductase."

The FMN content and molecular weight of old yellow enzyme has been reinvestigated by Theorell & Åkeson (255). Preparations with about the same FMN content (0.65 per cent) as those previously prepared by Theorell (256) were further purified by fractionation with alcohol at low temperatures, dialysis against ammonium sulfate and repeated recrystallization. The FMN content of the most highly purified preparations was found to be 0.877 per cent. Ultracentrifugation studies revealed that the enzyme has a molecular weight of approximately 104,000 and contains two FMN molecules.

A transfer reaction catalyzed by FAD-containing enzymes, but not by FMN-containing enzymes, has been described by Weber & Kaplan (257).

The reaction involves electron transfer from DPNH or TPNH to the acetylpyridine analogues of DPN or TPN. The enzymes exhibit specificity toward both the donor pyridine nucleotide and the acceptor analogue.

In studies on bacterial resistance to chlortetracycline (Aureomycin), Saz & Martinez (258) compared the nitrate reductase of a chlortetracycline-sensitive strain of *E. coli* with that of a strain resistant to chlortetracycline. The enzyme from the resistant strain contained firmly bound conjugated flavin (chiefly FMN), while the enzyme from the sensitive strain contained easily dissociable flavin and was readily reactivated by FMN.

#### INOSITOL

The synthesis, chemistry, and configuration of the inositols, inososes, quercitols, and inosamines have been comprehensively reviewed by Angyal (259).

Eagle *et al.* (260) have extended their original observations (261) on the requirement for *myo*-inositol (*meso*-inositol) of cells in tissue culture. *Myo*-inositol was found to be an essential growth factor for each of the eighteen human cell lines tested, whether normal or malignant, and for one of two lines of mouse cells. With two lines of human cells, HeLa and J-111, more thorough dialysis of the serum used in the medium was needed to demonstrate the inositol requirement. Geyer & Chang (262) have also shown a requirement for inositol by HeLa and human conjunctival cells. Many compounds, structurally related to *myo*-inositol, have little or no growth promoting activity for the human liver, intestinal and conjunctival cells tested (260). Phytic acid, and an inositol monophosphate derived from phytic acid, have slight growth-promoting activity, averaging 4 and 18 per cent that of free inositol. An inositol monophosphate, derived from liver, is much more effective than the plant-derived monophosphate, with about 75 per cent the activity of free inositol. A sample of *myo*-inosose had slight activity, presumably due to its known contamination with free inositol. Seven of the eight known inositol isomers had no activity (*allo*-inositol was not tested), nor did an inositol plant phosphatide, or several compounds structurally related to inositol. Haff & Swim (263) found no inositol requirement for a rabbit fibroblast, strain RM3-73, on a medium containing dialyzed horse serum. Although further dialysis may have shown an inositol requirement, it is also possible that this rabbit fibroblast, like the mouse fibroblast, L strain (260), does not require inositol.

Free *meso*-inositol (but not combined *meso*-inositol) was found in the lens of several species of animals, ranging in concentration from about 50 mg. per 100 gm. of lens in the rat to about 500 mg. per 100 gm. of lens in the sheep and human (264). It is present in aqueous humour in much lower concentrations. The concentration in the lens is lower in the very young animal (cattle, rabbit) than in the adult of the same species. No *meso*-inositol could be found in the lens of a rabbit in which cataract was induced by means of x-rays.

Following the finding by Mann (265) of large amounts of inositol in the seminal vesicle fluid of the boar, Hartree (266) studied the metabolism of inositol in the male reproductive organs by measuring the levels of inositol (*Kloeckera brevis* assay) in the seminal plasma of various species and in secretions from the accessory organs of the male reproductive tract. Boar seminal plasma contained 600 to 700 mg. inositol per 100 ml. whereas that of the bull, human, rabbit, ram and stallion contained less than 100 mg. per 100 ml. These concentrations of inositol, mainly in the free state, are much higher than those found in other body fluids. Inositol is considered a characteristic component of seminal fluid, and the content is an index of the secretory activity of the seminal vesicles. There is also a parallelism between the levels of inositol and fructose in body fluids, reviving the idea of some metabolic relationship between these compounds.

Hawthorne & Hübscher (267) have described the isolation from guinea pig liver of a phosphate ester of inositol, which they believe occurs in the free state. The phosphate ester is similar to that obtained by mild alkaline hydrolysis of the monophosphoinositide isolated from liver by McKibbin (268). A comparison of chromatographic methods of purification of liver monophosphoinositide has been made (269). The lipides of peas (*Pisum sativum*, var. *Thomas Laxton*) are a convenient source from which relatively pure phosphatidyl inositol can be isolated by a simple procedure (270). The presence of a small amount of inositol phosphatide in hens' egg yolk, first shown by Malangeau (271) was confirmed in two laboratories (272, 273) using silica columns for isolation (274). Further work on the preparation and purification of inositol glycerol phosphatidic acid from wheat germ was described by Morelec-Coulon & Faure (275).

A microspectrophotometric method involving  $\text{HIO}_4$  oxidation of the purified inositol has been developed for determination of inositol in tissues and lipide hydrolysates (276). Methods for isolation and purification of inositol from yeast cells and measurement of inositol by the anaerobic enzymatic reduction of 2,6-dichloroindophenol by inositol dehydrogenase (from *Acetobacter suboxydans*), coupled with pig heart diaphorase, have been described by Charalampous & Abrahams (277). A method for degradation of inositol to glyoxylic and formic acids was devised for studies of inositol synthesis (278).

Synthesis of inositol by yeast in cultures containing glucose labeled with  $\text{C}^{14}$  in various positions, and  $\text{C}^{14}$ -labeled acetate, formate or formaldehyde plus unlabeled glucose, indicate that inositol is not synthesized directly by cyclization of glucose, but rather from smaller fragments derived from glucose (279). The condensation of a tetrose with a 2-carbon compound may be a possible biosynthetic mechanism.

Intraperitoneal administration of tritium-labeled inositol to rats led to significant incorporation of tritium in lipide-containing extracts of liver, kidney, lung, brain and pancreas with the highest specific activity in the alcoholic extract of kidney (280). *In vitro* incorporation by liver and kidney

slices and homogenates was also demonstrated. The rate of incorporation of *meso*-inositol-2- $H^3$  into phospholipides by pancreas slices and brain cortex slices is markedly increased by acetylcholine (281). Cytidine nucleotides are needed for incorporation of inositol into inositol lipides by guinea pig kidney mitochondria in the presence of ATP and  $\alpha$ -ketoglutarate (282). A high amount of labeling of inositol phospholipide has been observed in various tissues of the rat following administration of  $P^{32}$ -labeled orthophosphate (283), confirming results with tissue slices (284, 285). Moscatelli & Larner (286) found that, after intraperitoneal injection of  $C^{14}$ -labeled *myo*-inositol in fasting rats, approximately 25 per cent of the injected dose appeared as  $CO_2$  in 12 hours. Liver glycogen and tissue phospholipide were labeled to a much smaller extent. A soluble enzyme system from rat kidney was found which catalyzes the oxidative conversion of *myo*-inositol to glucuronic acid (287). The isolated glucuronic acid was optically inactive and appeared to be a racemic mixture.

Most of the inositol present in *Neurospora crassa* is present in bound form as phospholipide (288). This complex is not readily available as a source of inositol for the inositol-requiring mutant strain 37401. Inositol is used for formation of lecithin-like compounds during growth of *Schizosaccharomyces pombe* (289).

The *in vitro* effect of soybean phosphatides in increasing the electrophoretic migration velocity of  $\beta$ -lipoprotein in human serum (290), and the effect on blood coagulation when given intravenously in the rabbit (291) may be related mainly to the inositol phosphatides present.

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## FAT-SOLUBLE VITAMINS<sup>1,2</sup>

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### VITAMIN A<sup>3</sup>

An excellent book, *Vitamin A*, by Moore (1) reviewed the biochemistry, physiology, and nutrition of this vitamin through 1955.

*Synthesis, chemistry, and biosynthesis.*—An improved procedure for the total synthesis of pseudoionone replaces the dependence of the commercial production of vitamin A and  $\beta$ -carotene on citral extracted from lemon grass [Kimel *et al.* (2)]. Another approach to  $\beta$ -carotene syntheses involved reaction of a C<sub>12</sub>-dienol ether "centerpiece" with two  $\beta$ -C<sub>14</sub>-acetal "tailpieces" followed by hydrolysis to yield the intermediate  $\beta$ -C<sub>40</sub>-diketone [Isler *et al.* (3)].  $\beta$ -Carotene-6,6'-C<sub>2</sub><sup>14</sup> was prepared by Würsch & Schwieter (4), employing a C<sub>19</sub>-aldehyde-6-C<sup>14</sup>, by procedures previously described by Inhoffen and by Isler. 11,11'-Di-*cis*- $\beta$ -carotene has the same structural relationship to  $\beta$ -carotene as 4-mono-*cis*-vitamin A aldehyde (neoretinene-*b*) has to the all-*trans* isomer. Isler *et al.* (5) synthesized this carotene isomer by procedures described above (3). A series of "hydrocarotenes" were prepared by stepwise catalytic hydrogenation of natural carotene (6). Syntheses of the other carotenoids will not be reviewed.

Instability of carotenoid pigments on exposure to  $\gamma$  radiation is considered by Lukton & Mackinney (7) to be dependent on the extent to which free radicals or peroxides formed in the surrounding medium are available for reaction. Films in the solid state were found to be remarkably stable.

Vitamin A was synthesized by rearrangement of the retro C<sub>15</sub>- and C<sub>20</sub>-acid chlorides to the normal system of double bonds [Huisman *et al.* (8)]. Both all-*trans*-vitamin A and the neo isomer were prepared by this scheme. By the selective formation of complexes of phenolic compounds with all-*trans*-vitamin A aldehyde, the all-*trans* isomer was separated from mixtures of vitamin A aldehydes containing the 2-*cis*, 2,6-di-*cis* and 6-*cis* isomers [Benton & Robeson (9)]. Use of PtO<sub>2</sub> by Karrer & Hess (10) for the rapid catalytic oxidation of vitamin A to the aldehyde led to contamination with

<sup>1</sup> The survey of the literature pertaining to this review was completed on October 1, 1957. Because of space limitations, the essential fatty acids are not reviewed.

<sup>2</sup> Communication No. 243 from the Biochemistry Department of Distillation Products Industries.

<sup>3</sup> The numbering system for the carotenoids corresponds to that officially adopted [Chem. Eng. News, 24, 1235 (1946); see also Goodwin, T. W., *Ann. Rev. Biochem.*, 24, 497 (1955)]. For vitamin A, the terminal alcoholic carbon of the side chain is assigned the number 1 in agreement with *Chemical Abstracts* [see 1955 Subject Index] and most previous reviewers [Quaife, M. L., *Ann. Rev. Biochem.*, 23, 215 (1954); Boyer, P. D., *Ann. Rev. Biochem.*, 24, 465 (1955); and Kodicek, E., *Ann. Rev. Biochem.*, 25, 497 (1956)].

*cis* isomers, limiting its usefulness when maintenance of known isomeric composition is desired.

Biosynthesis of the carotenoids in small quantities has been previously reported for many strains of fungi, *Phycomyces blakesleeanus* being the principal test organism. Barnett *et al.* (11) found that the production of  $\beta$ -carotene by *Choanephora cucurbitarum* was increased fifteenfold in combined cultures of "+" and "-" mating types compared with either type cultured separately. Hesseltine & Anderson (12) extended these findings to other members of the *Choanephoraceae*. Anderson *et al.* (13), using *Blakeslea tri-spora*, added 22 p.p.m. of  $\beta$ -ionone to the fermentation medium and further enhanced carotene production to a level of 3300  $\mu\text{g./gm.}$  of dry mycelium. Efficient utilization of chemical intermediates in carotene biosynthesis by fungi may soon be economically practical.

*Analysis.*—It has not been generally realized that a unit of vitamin A as determined by official procedures is not the same in the United States as it is in other countries. This discrepancy resulted in part from use of different solvents and other procedural differences discussed elsewhere (14, p. 76). The Vitamin Commission of the International Union of Pure and Applied Chemistry undertook standardization of vitamin A assays (15). They accepted the U.S.P. XV procedure with certain changes in the constants in the Morton and Stubbs equation and adopted a conversion factor of 1830 for vitamin A alcohol in isopropanol (16). Recently, the United States Pharmacopoeia agreed to follow the proposals of IUPAC (17). In effect, a unit of vitamin A determined by the new official procedure will be about 6.6 per cent less [3.7 per cent for conversion factors, 2.9 per cent for correction factors] than a unit determined by the original U.S.P. XV assay. A milestone in vitamin A analysis has thus been reached with international uniformity of official assay procedures.

In addition, a new U.S.P. Vitamin A Reference Solution was recently introduced. The older Reference Standard (Lot H) with a defined potency of 10,000 U.S.P. units/gm. had gradually deteriorated. The newly issued U.S.P. Vitamin A Acetate Reference Solution, a solution of synthetic all-*trans*-vitamin A acetate in vegetable oil, has a defined potency of 100,000 U.S.P. units/gm. (18). A unit of vitamin A as defined by these two standards can differ by up to 7 per cent in comparative assays.

Procedures for the determination of vitamin A were comprehensively reviewed by Embree *et al.* (14). Sorrels & Reiser (19) separated numerous fat-soluble materials on silicic acid-impregnated glass-fiber filter paper. However, decomposition of the more unstable compounds, such as vitamin A alcohol, limits its usefulness. Lagoni & Wartman (20) employed a novel chromatogram, using a layer of alumina approximately 0.5 mm. deep on a glass plate. When the materials were added dropwise, the compounds separated into concentric bands. Vitamin A dissolved in methyl alcohol was determined by a polarographic procedure (21). The photometric procedure was discussed further by De (22). Specific procedures have been devised for



various biological materials, such as feces (23, 24), plasma (25, 26), liver (27), and milk (28). Previously accepted procedures for the analysis of vitamin A in mixed feeds have been complicated by the introduction of various forms of stabilized vitamin A. Such preparations often contain wax, gelatin, hard fat, or organic polymers. Current difficulties were comprehensively surveyed by Parrish (29). Procedures involving either total saponification or extraction using a surfactant appear to offer the best possibility of success. Saponification followed by chromatography on weakened alumina gave reasonably good recoveries with various forms of stabilized vitamin A [Kyrning (30)]. Another modification for the determination of vitamin A in foods employed tandem column chromatography on alumina [Morgareidge (31)].

Chemical procedures for the separation and identification of the six geometric isomers of vitamin A have not yet been developed. Barnholdt (32) separated neovitamin A<sub>1</sub> and all-*trans*-vitamin A<sub>1</sub> by chromatography on alumina. In the same laboratory (33) perch liver oil was chromatographed on dicalcium phosphate. All-*trans*-vitamin A<sub>2</sub> was identified and a fraction was assumed to be neovitamin A<sub>2</sub>. On a calcium phosphate column, elution was in the order: neovitamin A<sub>1</sub>, neovitamin A<sub>2</sub>, all-*trans*-vitamin A<sub>1</sub>, and all-*trans*-vitamin A<sub>2</sub>. Marine fish liver oils were analyzed spectrophotometrically and reported to contain about 25 per cent neovitamin A<sub>1</sub> and 10 per cent vitamin A<sub>2</sub> (34).

Chemical procedures for the determination of  $\beta$ -carotene were comprehensively surveyed by Bickoff (35) and Booth (36). Absorption spectra of  $\beta$ -carotene crystals suspended in water by supersonic vibration were determined (37). Rabourn & Quackenbush (38) found substantial differences in the wavelength of maximum spectral absorption of  $\beta$ -carotene in various solvents. These findings emphasize the necessity of defining both temperature and solvent used for spectral identification of carotenoids. Rabourn & Schall (39) proposed a solution of  $\beta$ -carotene in purified white mineral oil as a reference solution for collaborative work. Procedures for the determination of carotene in feces (40) and feeds (41, 42) have appeared.

*Bioassay and biopotency.*—Bioassay procedures for vitamin A were standardized by Embree *et al.* (14) so that the three bioassays of primary importance, growth, liver-storage, and vaginal-cornification, could be run under standard conditions on the same diet. In growth bioassays, male rats grew better than did females at daily intakes of 2 I.U. and above [Guerrant (43)]. This inherent characteristic was not related directly to the initial body weight.

The liver-storage bioassay for vitamin A must be performed under carefully controlled conditions. The dietary fat level has no influence on vitamin A liver storage in rats (44). Using steers, Erwin *et al.* (45) confirmed that dietary fat did not modify liver storage of vitamin A, but its presence increased liver carotene. Green *et al.* (46) fed cholesterol simultaneously with vitamin A and observed no influences on liver storage of vitamin A in fe-

males. In male animals, depression of liver vitamin A was as great as 46 per cent for young and 16 per cent for adult males. Antioxidants in the diet increased liver storage of vitamin A (47). Harms *et al.* (48) found that the substance in condensed fish solubles which enhanced vitamin A storage in chick livers was acid-stable, alkali-unstable, and adsorbable on anion exchange resins. Identification of this substance is awaited with interest. Physiological stresses tend to lower vitamin A liver stores. Liver vitamin A was substantially decreased after rats had been subjected to exhausting physical exertion [Berger & Dietl (49)]. When the sympathetic center was stimulated, liver vitamin A decreased by about 40 per cent after only 60 min. [Nasu (50)]. Cold stimulation did not have this effect.

The biological assay of vitamin A based on the appearance of vaginal cornification in vitamin A depleted rats was extended and modified. Murray & Campbell (14, p. 64) and Clarke & Todd (51) independently reported that a straight line is obtained by plotting log dose against the number of days from dosing to repletion rather than against the log of the number of days as originally developed by Pugsley. Using this procedure, both laboratories found excellent precision in routine bioassays.

In a novel bioassay procedure reported by Gordon & Machlin (52), depleted chicks were placed on diets containing vitamin A for a 3-week growth period. After being returned to a vitamin A-free diet, time of survival was noted. This bioassay procedure is applicable to low levels of dietary vitamin A which cannot be determined chemically.

The biological potency of  $\beta$ -carotene is still a subject of considerable disagreement between various investigators. Barnett & Espoy (53) reported pure all-*trans*- $\beta$ -carotene from carrots to have a biopotency of 2,200,000 to 2,500,000 U.S.P. units/gm. rather than the assigned value of 1,667,000 units/gm. They suggested that the International Reference carotene is not pure all-*trans*- $\beta$ -carotene and that the bioassay techniques used may favor the utilization of vitamin A over provitamins A. Contrariwise, Marusich *et al.* (54) found that synthetic all-*trans*- $\beta$ -carotene averages 1,730,000 I.U./gm., in excellent agreement with the assigned value. They failed to confirm previous reports of enhanced activity of  $\beta$ -carotene dissolved in cottonseed oil or margarine (55). Clarke & Todd (51) bioassayed  $\beta$ -carotene by the rat vaginal-cornification procedure and found it to be 43 per cent as active as all-*trans*-vitamin A. The biological availability of  $\beta$ -carotene in a food or feed is of prime importance to nutritionists. Employing liver storage of vitamin A in calves, Rousseau *et al.* (56) found that at a level of 60  $\mu$ g./lb. wt./day,  $\beta$ -carotene was only one sixth as active as vitamin A. When the level was increased to 180  $\mu$ g., the ratio of activities was reduced to 1/12 and with a daily intake of 540  $\mu$ g.  $\beta$ -carotene was approximately 1/22 as active as vitamin A. Heat-treated alfalfa produced about 50 per cent less liver storage of vitamin A in chicks than sun-cured or steam-blanching alfalfa (57). The liver-storage procedure was used to determine the biological activities of the provitamins A (58).

Evaluation of the biological potency of derivatives of  $\beta$ -carotene was continued. Fazakerley & Glover (59) found that the  $C_{25}$  acids,  $\beta$ -12'-apo-carotenoic acid and  $\gamma$ -15-hydroxy-retinylacetic acid, were approximately equivalent to vitamin A in growth-promoting activity, whereas the  $C_{22}$  acids,  $\beta$ -14'-apo-carotenoic acid and 15-hydroxy-retinyltiglic acid, were only 1/20 and 1/5 as active, respectively, as vitamin A. Isler *et al.* (60) showed that 3,4,3',4'-bis-dehydro- $\beta$ -carotene had only about 40 per cent the vitamin A activity of  $\beta$ -carotene. The  $C_{41}$ ,  $C_{42}$ , and  $C_{46}$  hydrocarbons all had considerable biological activity both for growth and liver storage (61). Astaxanthin esters promoted nearly normal growth in young rats deprived of vitamin A, but showed only partial activity in supporting reproduction (62). When fish deprived of vitamin A were fed astaxanthin, both vitamin  $A_1$  and vitamin  $A_2$  were identified in livers and eyes (63).

Six geometric isomers of vitamin A have now been biologically assayed by Ames *et al.* (64) both as acetates and as aldehydes. Table I includes additional data from the laboratories of Distillation Products obtained subsequent to the original reports (65, 66). Clarke & Todd (51), employing the vaginal-cornification rat bioassay, reported the relative potency of neovitamin A to be 68 per cent that of all-*trans*-vitamin A, in excellent agreement with the data in Table I. Fractions containing high levels of *cis* isomers (mostly hindered 4-mono-*cis*-vitamin A) had relative potencies of 36 and 45 per cent.

TABLE I  
BIOLOGICAL POTENCY OF THE GEOMETRIC ISOMERS OF VITAMIN A

Isomer	Trivial Name	Isomeric Acetates		Isomeric Aldehydes	
		Biopotency	Relative Molar Biopotency	Biopotency	Relative Molar Biopotency
		units/gm.	%	units/gm.	%§
all- <i>trans</i> -		(2,907,000)‡	(100)	3,050,000	91
2-mono- <i>cis</i> -	neo*	2,190,000	75	3,120,000	93
6-mono- <i>cis</i> -	iso-a†	607,000	21	637,000	19
2,6-di- <i>cis</i> -	iso-b†	688,000	24	581,000	17
4-mono- <i>cis</i> -	neo-b†	690,000	24	1,580,000	47
2,4-di- <i>cis</i> -	neo-c†	428,000	15	1,030,000	31

\* According to Robeson, C. D., and Baxter, J. G., *J. Am. Chem. Soc.*, **69**, 136 (1947).

† According to Wald [see Blaxter, K. L., *Ann. Rev. Biochem.*, **26**, 275 (1957)].

‡ By definition.

§ 3,357,000 units/gm. = 100%.

|| 2,4-Di-*cis*-vitamin A alcohol and acetate furnished by Oroshnik [see Oroshnik, W., *J. Am. Chem. Soc.*, **78**, 2651 (1956)].

Reports on vitamin A-active materials, not giving chemical reactions like vitamin A, were well summarized by Lowe & Morton (67). An early report of Ernster *et al.* (68) that vitamin A was present in yeast incubated in an O<sub>2</sub> atmosphere was not verified by Heaton *et al.* (69) and Redfearn (70). These findings eliminated the only reported exception to the rule that preformed vitamin A occurs only in animal tissues.

**Metabolism.**—Vitamin A-2-C<sup>14</sup> was injected by Wolf *et al.* (71) and about 5 per cent of the dose appeared as expired C<sup>14</sup>O<sub>2</sub> in 24 hr. Kidneys showed little radioactivity, but urinary excretion of water-soluble C<sup>14</sup>-compounds was noted. Liver storage of vitamin A was considerably lower than reported previously (14) and excretion data showed tremendous variation between the two rats studied. Aqueous dispersions of vitamin A were injected intravenously into rats by Pollard & Bieri (72). Extensive destruction of vitamin A was noted, apparently by some factor in erythrocytes. Liver storage of vitamin A was considerably below that found by others with orally administered oil solutions of vitamin A (14). Squibb *et al.* (73) reported somewhat higher serum vitamin A levels following intramuscular injection of aqueous dispersions than with oral feeding of aqueous dispersions or oil solutions. Absorption of vitamin A was shown by Tanaka (74) to be extremely rapid. Vitamin A appeared in the retinas of vitamin A-deficient rats within 3 hr. after its oral administration.

Vitamin A is undoubtedly transported in the blood as a protein complex. Earlier studies were extended by *in vitro* synthesis of vitamin A-protein complexes and identification of such complexes in the blood of man and animals (75). Ganguly *et al.* (76, 77) continued their investigations of vitamin A esterases in various organs. Neither blood nor liver hydrolyzed naturally-occurring vitamin A esters, whereas pancreas did. They proposed that the vitamin A ester of circulating blood originates in the liver and is hydrolyzed by other tissues. High *et al.* (78) found that *in vivo* and *in vitro* hydrolysis of vitamin A esters was a function of chain length of the ester. Both hydrolysis of esters and esterification of vitamin A alcohol occurred fairly rapidly following intravenous injection. Takai *et al.* (79) again indicated that the free form is the most significant indicator of vitamin A status.

In view of (a), the natural occurrence of isomers of vitamin A in addition to the all-*trans* form, (b), the specific activity of 4-mono-*cis*-vitamin A aldehyde in the eye, and (c), the all-*trans*-isomer possessing the greatest biological potency for growth, liver storage, and vaginal cornification, the *in vivo* isomerization of vitamin A esters was of considerable interest. Hubbard (80) prepared an enzyme from cattle retinas catalyzing the interconversion of all-*trans*-vitamin A aldehyde and the 4-mono-*cis* isomer. Ames *et al.* (64) reported that the rat can interconvert orally administered isomers and store a mixture of all-*trans* and less active *cis* isomers in the liver. Even 6-*cis* isomers of low biopotency were isomerized in part to all-*trans*-vitamin A. An equilibrium mixture of isomers was stored in the liver independent of the isomer fed. Both the 4-mono-*cis* and 2,4-di-*cis*-vitamin A aldehydes were about

twice as active biologically as the corresponding acetates (see Table I), supporting earlier conclusions (66) that the aldehyde group labilizes the 2-3 and 4-5 double bonds enabling the rat to isomerize the aldehydes prior to absorption.

Murray & Campbell (81) indicated that the effect of chlortetracycline (Aureomycin) in enhancing the activity of vitamin A was not due to increased intestinal absorption. The effects of stress on vitamin A serum levels were investigated. In adrenalectomized animals the serum vitamin A levels were not changed (82). Sterile abscesses resulted in a lowering of serum vitamin A levels [Kaiser (83)]. This occurred regardless of whether the animals were normal, adrenalectomized, or dosed with cortisone or hydrocortisone. Factors other than adrenal function apparently are involved in maintaining serum vitamin A levels.

Lowe & Morton (67) have surveyed the literature through 1954 on the conversion of  $\beta$ -carotene to vitamin A. Studies on the intestinal conversion of  $\beta$ -carotene to vitamin A by Greenberg (84) utilized differential fluorescence examination of frozen tissue sections. Vitamin A appeared first within the intestinal lumen adjacent to the tips of the villi, and only later within the intestinal mucosa, suggesting an extra-cellular process. On the other hand, Worker (85) concluded that intravenously administered aqueous dispersions of  $\beta$ -carotene can be converted into vitamin A by many organs. The metabolism of  $C^{14}$ - $\beta$ -carotene in the rat has been reported from three laboratories. Fishwick & Glover (86) found that 50 to 70 per cent of the dose was not absorbed, 2 per cent was eliminated as  $C^{14}O_2$ , and approximately 30 per cent was stored in the liver as vitamin A.  $C^{14}$ - $\beta$ -carotene was degraded to yield intermediary metabolites resulting in  $C^{14}$ -sterol and  $C^{14}$ -fatty acid fractions as well as vitamin A. These data indicated that  $C^{14}$ - $\beta$ -carotene was attacked at other than the central double bond but not solely by  $\beta$ -oxidation. Krause & Sanders (87) found 70 per cent absorption of  $C^{14}$ - $\beta$ -carotene, 5 per cent excretion as  $C^{14}O_2$  in 24 hr. and substantial quantities of  $C^{14}$ -fatty acids. About 40 per cent of the absorbed  $C^{14}$  was not accounted for. The highest radioactivity per unit weight of organ appeared in adrenals following oral administration of  $C^{14}$ - $\beta$ -carotene [Willmer & Laughland (88)]. Redfearn (89) fed a  $C_{25}$ -homologue of vitamin A and found that the  $\beta$ -methyl group in the  $C_{25}$ -alcohol did not completely inhibit oxidation to vitamin A, thus failing to substantiate the hypothesis that the  $\beta$ -methyl group of vitamin A results in termination of progressive oxidation of  $\beta$ -carotene. On feeding a mixture of  $\alpha$ - and  $\beta$ -carotene, McAnally & Szymanski (90) found an ultraviolet absorption maximum corresponding to the  $\alpha$ -ionone analogue of vitamin A in the liver unsaponifiable fraction. These results indicated that  $\alpha$ -carotene, like  $\beta$ -carotene, is metabolized by stepwise oxidation rather than by attack at the central double bond.

*Function and metabolic effects.*—The systemic mode of action of vitamin A has been reviewed by Lowe & Morton (67). Wolf *et al.* (91) determined the effects of vitamin A deficiency on the ability of the rat to incorporate labeled

intermediates into a number of metabolic products. Vitamin A is apparently not involved in the tricarboxylic acid cycle nor in cholesterol or fatty acid biosynthesis. Incorporation of acetate-1-C<sup>14</sup>, lactate-1-C<sup>14</sup>, and glycerol-1,3-C<sup>14</sup> into liver glycogen was greatly reduced in vitamin A deficiency, whereas incorporation of glucose-1-C<sup>14</sup> was not modified. Vitamin A apparently is involved, directly or indirectly, in the reversal of glycolysis between the triose and the glucose stage. Agnew & Mayer (92) also rejected an active role for vitamin A in fatty acid synthesis. Brüggemann & Niesar (93) observed a decrease in DNA, but not RNA in several organs during vitamin A deficiency. Administration of vitamin A resulted in a significant rise in the nucleic acid level, suggesting its involvement in nucleic acid metabolism. Endogenous respiration in livers of vitamin A-deficient animals was significantly increased, whereas succinoxidase activity was not changed [Redfearn (94)]. Cytochrome-*c* reductase was markedly decreased in vitamin A deficiency, suggesting that vitamin A, along with vitamins E and K, may be implicated in electron transport systems.

Underdahl & Young (95) demonstrated increased protection against influenza virus with vitamin A supplementation of vitamin A-deficient mice, confirming the reported action of vitamin A in increasing resistance to infection. Vitamin A increased the effectiveness of ethylenediamine tetra-acetic acid in removing lead<sup>210</sup>, previously deposited in the bone (96). Keratinization which develops during vitamin A deficiency was explained on the basis that vitamin A may normally suppress copper-catalyzed oxidation of thiol groups to disulfides (97).

The well-known function of vitamin A as a precursor for rhodopsin, a visual pigment, was further clarified by isolation of 4-mono-*cis*-vitamin A aldehyde (neoretinene-*b*) and demonstration that it is the specific hindered *cis*-polyene required for the formation of the visual pigment [Brown & Wald (98)]. The visual pigments have been reviewed by Dartnall (99). 4-Mono-*cis*-vitamin A aldehyde was found to be a component of the visual pigment in the lobster (100, 101), and in the euphausiid crustacean (102).

*Deficiency.*—Vitamin A deficiency was produced in kittens by Gershoff *et al.* (103) with symptoms similar to those reported for other species of animals. The deficiency symptoms were not prevented by  $\beta$ -carotene, indicating limited conversion of  $\beta$ -carotene to vitamin A in the cat. Vitamin A deficiency in chicks was produced by the incorporation of a finely powdered absorbent in the ration (104). Reproductive abnormalities due to vitamin A deficiency were reported from several laboratories. Vitamin A deficiency in cattle produced disturbances in ovarian function, resulting in infertility [Jaskowski *et al.* (105)]. In the rabbit, vitamin A deficiency reduced the litter size and increased the mortality of young. Injection of progesterone did not affect litter size, but improved viability of the young (106). Suboptimal  $\beta$ -carotene intake in cattle produced a high incidence of stillborn calves with the appearance of hydrocephalus, degeneration of the optic nerve, and reproductive abnormalities in the calves that survived (107). Watt & Barlow (108)



attributed the probable cause of microphthalmia in piglets to vitamin A deficiency. Millen & Woollam (109) found hydrocephalus in the young of vitamin A-deficient rabbits resulted from an increased production of cerebrospinal fluid. In similar studies on vitamin A-deficient chicks, increased cerebrospinal fluid pressures were found (110).

*Hypervitaminosis-A*.—Hypervitaminosis-A in humans was reviewed by Breslau (111). Hillman (112) induced hypervitaminosis-A in himself with daily oral doses of one million units of vitamin in aqueous emulsion. Plasma vitamin A levels rose to approximately 700 and 1800  $\mu\text{g.}$  per cent over two experimental periods of 14 and 25 days, but dropped rapidly to normal levels when supplementation was discontinued. Clinical manifestations included headache, cheilosis, generalized desquamation, and fragility of the fingernails. Bone abnormalities are characteristic of hypervitaminosis-A. Fell *et al.* (113) found that hypervitaminosis-A inhibited sulfur metabolism of bone rudiments grown *in vitro*, and suggested that under the influence of excess vitamin A cartilage cells form soluble sulphated mucopolysaccharides, instead of the normal chondroitin sulphate, resulting in dissolution of the existing matrix. Induction of fetal abnormalities in rats by massive doses of vitamin A were both confirmed (114) and refuted (115). Symptoms of hypervitaminosis-A were potentiated by supplements of alfalfa meal and other succulents, desiccated liver, yeast, and chlortetracycline [Ershoff *et al.* (116)]. Dietary supplementation with various flavonoids increased symptoms of hypervitaminosis-A in rats (117). Alkaline phosphatase levels in a number of organs in young rats rose progressively with increasing amounts of vitamin A in the diet (118). All-*trans*- $\beta$ -carotene fed at a level of 1 gm./kg. body weight for 4 months had no adverse effect on either rats or rabbits [Isler *et al.* (60)].

#### VITAMIN D

*Synthesis and biopotency*.—Inhoffen *et al.* investigated *cis* and *trans* forms of vitamin D and corresponding model systems. Isomerization of 5,6-*cis*-vitamin D<sub>2</sub> with BF<sub>3</sub> and iodine yielded a mixture of 5,6-*trans*-vitamin D<sub>2</sub>, isovitamin D<sub>2</sub>, and isotachysterol<sub>2</sub> (119). 5,6-*Trans*-vitamin D<sub>2</sub> synthesized from a C<sub>27</sub>-ketone via a C<sub>21</sub>-hydrindan-ketone had only 0.8 per cent the anti-rachitic activity of 5,6-*cis*-vitamin D<sub>2</sub> (120). After irradiation, *trans*-vitamin D<sub>2</sub> was increased sixfold in biopotency, but this was not considered unequivocal evidence of partial synthesis of the *cis* isomer. Ultraviolet radiation successfully converted the 5,6-*trans*-vitamin D<sub>2</sub> to the 5,6-*cis* isomer (121). Oxidative decomposition of vitamin D<sub>3</sub> with ozone yielded a *trans*-C/D-hydrindan alcohol ("abbau" alcohol) and further oxidation gave the corresponding ketone (122). Dihydrotachysterol<sub>3</sub> was prepared by Werder (123) by hydrogenation of vitamin D<sub>3</sub>-3,5-dinitrobenzoate, and by van de Vliervoet *et al.* (124) by reduction of tachysterol<sub>3</sub>. The latter reported dihydrotachysterol<sub>3</sub> to be twice as active as dihydrotachysterol<sub>2</sub> in raising blood calcium levels. Schubert (125, 126) prepared pure dihydrotachysterol<sub>2</sub>



(dihydrovitamin D<sub>2</sub> II) by catalytic hydrogenation of vitamin D<sub>2</sub>. By reduction of vitamin D<sub>2</sub> with sodium butylate, Werder (127) prepared a series of dihydrovitamins D<sub>2</sub>. Dihydrotachysterol<sub>2</sub> exhibited no antirachitic properties at a level of 12.5 µg./day and no toxicity at a level of 200 µg./day. Dihydrovitamin D<sub>2</sub> IV was effective in rats at a level of 500 µg./day, and a daily dose of 2 mg./20 gm. body weight was toxic in mice. Two wholly synthetic vitamin D homologues, the 2,1'-*cis* and 2,1'-*trans* isomers of 1-cholestanylidene-2-(5'-methoxy-2'-methylene-1'-cyclohexylidene)-ethane, were synthesized by Milas & Priesing (128). The *cis* isomer approached vitamin D<sub>2</sub> in activity whereas the *trans* isomer was much less active. The corresponding 1-cyclohexylidene derivative was also synthesized (129).

Raoul *et al.* (130, 131) have isolated "ketone 250," a highly antirachitic substance from plant material [see Blaxter (132)]. An uncharacterized antivitamin D was isolated from leaves by the same workers (133). Tested on chicks, it reduced the antirachitic potency of vitamin D<sub>3</sub>. A water-soluble rachitogenic factor was found in raw pig's liver [Coates & Harrison (134)].

*Analysis.*—A procedure for the routine determination of vitamin D described by Mulder *et al.* (135), employed a combination of maleic anhydride addition and colorimetric determination using SbCl<sub>5</sub>. Results compared favorably with results of rat and chick bioassays, but interference by vitamin A was not eliminated. Shaw *et al.* (136) developed methods suitable for the determination of the composition of complex irradiation mixtures. After chromatography on activated alumina, it was analyzed by ultraviolet spectroscopy using the reaction with SbCl<sub>5</sub>. Weits (137) indicated that present chemical methods are not suitable for the estimation of vitamin D in forages. Extracts for biological assays were prepared by boiling the forage in 10 per cent alcoholic KOH prior to extraction, followed by chromatography on alumina.

*Metabolism, function, and metabolic effects.*—With a histochemical method employing fluorescence, Araki *et al.* (138) found vitamin D in the mucous epithelium of the intestinal tract within two to three hours after an oral dose. Vitamin D appeared in the liver within four hours after administration. It was also present in kidneys, lung, adrenal cortex, and adipose tissue.

Vitamin D markedly increased absorption and retention of Ca<sup>45</sup> given orally to chicks but had no effect on intramuscularly injected Ca<sup>45</sup> [Keane *et al.* (139)]. It was concluded that the effect of vitamin D on calcium metabolism was primarily on absorption from the intestinal tract. Gershoff & Hegsted (140) fed diets containing various Ca/P ratios to chicks and found that the ratio had no significant effect on intestinal absorption of Ca<sup>45</sup> if adequate vitamin D was present. Without adequate vitamin D, calcium absorption was inversely proportional to the Ca/P ratio. Cramer & Steenbock (141) found that increasing the calcium intake in the presence of vitamin D changed the negative calcium balance induced in rats by a low-phosphorus diet to a positive one. Growth was decreased while the calcium content in blood and bone ash was increased, supporting the theory that the

decrease in growth induced by addition of vitamin D was effected by differential shunting of phosphorus from the metabolic pool to bone instead of to soft tissues. Bauer *et al.* (142) studied the effect of vitamin D on bone accretion and resorption in children with vitamin D-deficient rickets and vitamin D-resistant rickets. Low accretion rates were found in the former, but the rates rose to normal with administration of vitamin D. By contrast, the children with vitamin D-resistant rickets showed approximately normal accretion rates which rose markedly on administration of large doses of vitamin D. Pickering *et al.* (143, 144, 145) investigated the effect of vitamin D deficiency on chemical growth dynamics in the skeleton of young rats and stressed that in young growing rats, examination of one aspect of the skeleton does not permit conclusions to be drawn about any other aspect at the same age. On administration of vitamin D,  $\text{Ca}^{45}$ , and  $\text{P}^{32}$  to rachitic rats, Scheer & Kautz (146) found maximum  $\text{P}^{32}$  incorporation into bone within one day following the administered dose, whereas  $\text{Ca}^{45}$  showed maximum incorporation on the fifth day. Disturbances of mineralization in teeth appeared in vitamin D-deficient dogs (147). Gershoff *et al.* (148) reported that more severe rickets was produced in cats on vitamin D-deficient diets with a Ca/P ratio of 1.3/1 than with a ratio of 4/1. Spontaneous healing indicated a low vitamin D requirement in the young adult cat. Hiltbran *et al.* (149) found significantly more radium retention by animals receiving vitamin D than by untreated rachitic animals receiving a high calcium-low phosphorus diet.

De Luca & Steenbock (150) found increased plasma alkaline-phosphatase levels in rats on various vitamin D-free rations, but could not correlate plasma phosphatase levels with the severity of rickets. Löhr (151) found increased glucose-6-phosphatase in kidneys but not in livers of rachitic rats.

Burger *et al.* (152) implanted rachitic bone slices subcutaneously and found calcification in normal animals but none in rachitic animals. This difference was ascribed to a higher product of calcium times phosphorus concentrations in body fluids of control animals.

Dikshit (153) found that parathyroidectomy did not alleviate the effects of administration of small or large doses of vitamin D to rachitic rats. Elliott & Freeman (154) intensively studied calcium and citric acid metabolism in parathyroidectomized rats given vitamin D or a parathyroid extract. They concluded that the fundamental question as to whether the parathyroid gland directly or indirectly influences mobilization or metabolism of citric acid is still unanswered.

The relationship between vitamin D and citric acid metabolism was investigated at the enzymatic level by De Luca *et al.* (155, 156). Addition of vitamin D to rachitogenic or nonrachitogenic diets reduced the oxidation of citrate by kidney homogenates or mitochondria, but had no effect on oxidation of other intermediates in the Krebs cycle. This probably accounts for the increases in citrate content of certain tissues and in citraturia following vitamin D administration. While early attempts to demonstrate an *in*

*vitro* effect of vitamin D were unsuccessful, De Luca & Steenbock (157) recently reported that addition of vitamin D to a kidney mitochondrial preparation *in vitro* reduced citrate oxidation. However, the efficiency of phosphorylation coupled with oxidation was not effected.

*Hypervitaminosis.*—The toxic manifestations of massive doses of vitamin D are in part due to increased calcium absorption. Using  $\text{Ca}^{45}$  Conrad *et al.* (158, 159) found an increase in the absorption of calcium by cows during administration of vitamin D. Plasma calcium and phosphorus levels did not begin to rise until some days after absorption had increased. Blackburn *et al.* (160) reported differences in serum calcium, depending upon the calcium salt fed simultaneously with massive doses of vitamin D. Calcium carbonate induced higher serum calcium and more rapid mortality than calcium phosphate.

In hypervitaminosis-D in rabbits Donath *et al.* (161) found increases in serum cholesterol, alkaline phosphatase, calcium, and nonprotein nitrogen, and decreases in red-cell count. Scharfman & Propp (162) ascribed certain human anemias to high levels of vitamin D intake.

Increased absorption of calcium in hypervitaminosis-D led to calcification of tissues, as reported by Cole *et al.* (163) for cattle and by Blackburn *et al.* (160) for calves. Calcification of the vascular system was especially noticeable at the aorta base during vitamin  $\text{D}_3$  toxicity in calves. In addition to arterial lesions, Gilman & Gilbert (164) found kidney injury in rats and noted that the arterial lesions resembled those seen in severe kidney disease induced by other techniques. Arterial calcification occurs in some vascular diseases and thus de Langen & Donath (165, 166) simultaneously fed high levels of vitamin D and cholesterol. Such supplementation was more effective in producing atheromatosis in rabbits than cholesterol alone. Trueheart *et al.* (167) noted that the vascular disease induced by hypervitaminosis-D differs from the human disease in that it lacks an atheromatosis component. With superimposed hypercholesterolemia, simultaneous or sequential development of these two metabolic forms of vascular disease was observed in the same rabbit.

Cruikshank & Kodicek (168) found that treatment with cortisone acetate increased rather than decreased the effects of hypervitaminosis- $\text{D}_2$  in rats, thus failing to confirm the hypothesis that cortisone acts as a structural anti-metabolite toward vitamin D. Selye & Bois (169) studied calcification of the gastric mucosa in rats following vitamin  $\text{D}_2$  injections and reported that hydrocortisone acetate increased calcium deposits but deoxycorticosterone acetate did not. Arterial damage in rats on high levels of calciferol was reduced with cortisone therapy, whereas thyroxin or deoxycorticosterone acetate intensified the damage [Gilman & Gilbert (170)]. Massive doses of vitamin  $\text{D}_2$  increased serum levels of seromucoid in rats [Eisenstein & Groff (171)]. Certain hormones can modify, in part, the damage induced by hypervitaminosis-D.

## VITAMIN E

*Chemistry and biosynthesis.*—Further studies on the synthesis of vitamin E resulted in the preparation of phytol from ethyl levulinate (172) and isophytol from hexahydropseudoionone (173). Martius & Elingsfeld oxidized  $\alpha$ -tocopherol with iron salts (174) and found the "tocopheroxide" formed to be a quinone acetal (175). A tocopherol-like lipid with reducing properties and antihemolytic activity comparable to  $\alpha$ -tocopherol was isolated from yeast (176). Another unknown lipid with reducing properties was found (177) during chromatography of soybean oil. These lipides are probably not tocopherols, but their similar properties may interfere with chemical analyses.

Biosynthesis of tocopherols can occur in plants, but not in animals. In alfalfa the rate of synthesis was most rapid after maximum accumulation of carotene and chlorophyll. In sprouting seeds, some tocopherols were formed in the dark, but light stimulated the reaction (178). Interconversion of the tocopherols during the development of plants has been alluded to (179), but no data have appeared.

*Analysis.*—The separation and quantitative determination of each of the seven naturally occurring tocopherols is still under investigation. The determination of  $\alpha$ -tocopherol and partial separation and determination of the individual tocopherols were described by Bro-Rasmussen & Hjarde, employing column chromatography on secondary magnesium phosphate (180, 177). Carotenoids were not adsorbed and appeared in the petroleum ether eluate.  $\alpha$ - and  $\zeta$ -Tocopherols were quantitatively eluted with petroleum ether containing 2 per cent diethyl ether.  $\beta$ -,  $\gamma$ -, and  $\epsilon$ -Tocopherol were eluted with petroleum ether containing 4 per cent diethyl ether and  $\delta$ -tocopherol with 6 to 7 per cent diethyl ether. Appreciable losses occurred during saponification and extraction of products of low potency. For the differential determination of  $\alpha$ -tocopherol, this procedure appears simpler than the reversed-phase partition chromatographic procedure of Eggitt & Norris (181). Combination of the essentially quantitative saponification and extraction procedure of Eggitt & Ward (182) followed by chromatography on magnesium phosphate columns should give excellent results.

Use of paper chromatography for the separation of the tocopherols was thoroughly reviewed by Blaxter (132). Modifications employing paper impregnated with petroleum oil with an aqueous methanol solvent system (183), with 80 per cent ethanol (184), and with a mixture of ethanol, butanol, water, acetic acid, and petroleum (185) were reported.

The determination of  $\alpha$ -tocopheryl acetate added as a dietary supplement usually involved initial saponification (186, 180). This procedure lacks specificity, since  $\alpha$ -tocopherol must then be separated from the other tocopherols present. In a differential method developed by Ames & Tinkler (187), a solvent extract of the supplemented feed was passed through a Florex column, a portion of which had previously been treated with acidified

ceric sulfate, thus oxidizing and adsorbing naturally occurring reducing substances. The stable  $\alpha$ -tocopheryl acetate was quantitatively eluted from the column, saponified, and determined colorimetrically.

Tissue tocopherols estimated by a phosphomolybdic acid method gave somewhat higher results than other procedures (188). Hydrogenation was equally effective and more rapid than chromatography in eliminating carotenoid interference in plasma tocopherol determinations (189). Feldheim (190) found considerable variation in serum tocopherol values obtained by various procedures. Methods were reported for the determination of tocopherols in autoxidizing methyl esters of fatty acids (191), in plant matter (192), and in corn oil (193). The determination of tocopherols as a method for the detection of foreign fats in dairy products was subjected to collaborative study (194).

*Bioassay and biopotency.*—The liver-storage bioassay for vitamin E was further developed. Supplemented diets are fed to depleted animals for two to three weeks and the liver removed and analyzed for total tocopherols. Removal of artifacts by molecular distillation was shown by Bunnell *et al.* (195) to be an essential step. Since the liver storage of tocopherols in the chick is a linear function of the dose fed, Pudelkiewicz *et al.* (196) used the slope-ratio liver-storage procedure developed for vitamin A by Ames & Harris (197). Rousseau *et al.* (198) reported that in calves, lambs, and pigs, the liver tocopherol level was a linear function of tocopherol intake.

Preferential destruction of vitamin E in the diet, destruction following ingestion, and inherent lack of biological availability all tend to depress the true vitamin E level below chemically determined values. For the prevention of encephalomalacia, Singsen *et al.* (199) found that vitamin E in alfalfa was only partially utilized, whereas the vitamin E in wheat middlings was fully available. Scott & Nelson (200) reported, to the contrary, that the vitamin E in alfalfa was fully available for the prevention of exudative diathesis in chicks fed a diet high in Torula yeast. However, this syndrome is not specific for vitamin E [*vide infra*]. Bunnell (201) and Pudelkiewicz *et al.* (196), using a chick liver-storage bioassay, reported that approximately one-third of the vitamin E in alfalfa was available. The biological effectiveness of vitamin E can only be determined by bioassay, preferably by use of the animal eventually consuming the diet.

Bunyan *et al.* (202), using the rat anti-sterility bioassay, reported that  $\zeta$ -tocopheryl acetate was approximately half as active as *dl*- $\alpha$ -tocopheryl acetate. Recent investigations have indicated  $\zeta$ -tocopherol to be about one-third as active as the  $\alpha$ -form (203). Thus,  $\zeta$ -tocopherol joins  $\beta$ -tocopherol in possessing significant biological potency.  $\gamma$ -Tocopherol is only slightly active, and the mono-methyl-tocopherols ( $\delta$ -,  $\epsilon$ -, and  $\eta$ -) are probably without significant activity. Biopotencies of the various tocopherols are further complicated by the occurrence of both natural (*d*-) and synthetic (*dl*-) forms. Primarily on the basis of rat anti-sterility bioassays, *d*- $\alpha$ -tocopherol is officially accepted as being 1.36 times as effective as *dl*- $\alpha$ -tocopherol (204). In chicks,

using a liver-storage bioassay, Pudelskiewicz *et al.* (196), found that *d*- $\alpha$ -tocopheryl acetate was about 1.30 times as active as *dl*- $\alpha$ -tocopheryl acetate, in excellent agreement with the accepted value. The defined potencies for the unesterified  $\alpha$ -tocopherols are 1.1 I.U./mg. for the *dl*-form and 1.49 I.U./mg. for the *d*-form (204). Because of the inherent instability of unesterified tocopherols, these values must be used with extreme caution in estimating the biopotency of  $\alpha$ -tocopherol fed in borderline amounts in natural products or mixed feeds. Determination of the biological potency of other forms of vitamin E is urgently needed.

*Absorption, transport, and storage.*—The efficiency of absorption of orally fed tocopherols is not great (205). Plasma tocopherols in calves, lambs, and pigs increased at diminishing rates with tocopherol intake [Rousseau *et al.* (198)]. A similar response was noted in chicks (195). Serum tocopherol levels can also be modified by factors other than dose size. Postel (206) found inverse correlation with basal metabolic rate in humans.

Ingestion of tocopheryl esters leads to rapid hydrolysis of the ester and absorption of free tocopherols. However, Simon *et al.* (207) showed that in rats injected with *d*- $\alpha$ -tocopheryl-5-methyl- $C^{14}$ -succinate, the bulk of the material appearing in the liver after 24 hr. was unhydrolyzed ester. This is one of the first reports of unhydrolyzed tocopheryl esters appearing in body tissues. Of the radioactivity, 60 to 80 per cent appeared in the mitochondrial and microsomal fractions.

The bulk of the tocopherols stored in the body is found in the adipose tissue. An unsuspected species difference was reported by Mecchi *et al.* (208). Deposition of tocopherol in the carcass fat was much greater for the chicken than for the turkey.

*Metabolic effects.*—Because of its chemical structure,  $\alpha$ -tocopherol has long been postulated to be an integral part of enzymatic electron-transport systems. Previous studies by Nason & Lehman (209) revealed that crude preparations of DPN-cytochrome-*c* reductase could be inactivated by iso-octane extraction and reactivated by addition of either the extract or  $\alpha$ -tocopherol. This was not true for highly purified preparations of the enzymes. Similar findings were reported for the succinate-cytochrome-*c* reductase activity of a pig heart system by Morrison *et al.* (210) and Marinetti *et al.* (211). Slater *et al.* (212) found that  $\alpha$ -tocoquinone occurred in enzyme preparations in amounts commensurate with the enzyme activity. However, Donaldson & Nason (213) found that the lipid extract contained only 10 to 20 per cent of the total  $\alpha$ -tocoquinone occurring in the enzyme system, even though 90 per cent of the activity was lost on extraction. They purified the "lipide cofactor," tentatively identified it as a mixed glyceride of stearate, palmitate, and oleate, and proposed that it acted indirectly by releasing endogenous vitamin E to the "active site" of the enzyme. In a similar system, Marinetti *et al.* (214) found that the lipid possessing the greatest activity was a long-chain keto ester. While the evidence that vitamin E participates in the electron-transport system is suggestive, there is yet no evidence that it



undergoes oxidation and reduction as an integral part of an enzyme system.

$\alpha$ -Tocopheryl phosphate was reported (215) to inhibit reduction of ferric cytochrome-*c* by ascorbic acid or by cytochrome-*b*<sub>2</sub> and lactate, whereas  $\alpha$ -tocopherol had no such action. Rat diaphragm showed a reduction in respiration and an enhanced utilization of 5-hydroxytryptamine in the presence of  $\alpha$ -tocopheryl phosphate (216). When  $\alpha$ -tocopheryl phosphate was added, D-amino acid oxidase and amine oxidase of rat liver were stimulated (216). As stated previously (217),  $\alpha$ -tocopheryl phosphate is a nonspecific detergent, and inhibition of enzyme systems by this derivative should be interpreted with caution.

A defect in oxidative phosphorylation has been suggested in vitamin E deficiency, but its role is still not clarified. Carpenter *et al.* (218) indicated a significant lowering of P/O ratios in liver mitochondria. The enzymes involved in utilization of creatine phosphate also appeared to be modified in vitamin E deficiency. Phosphate transfer from creatine phosphate and phosphoglucomutase was decreased, but this occurred only with a simultaneous deficiency of both vitamin E and choline (218).

Tocopherol deficiency alters metabolism of various nitrogenous constituents. Dinning & Fitch (219) injected glycine-2-C<sup>14</sup> and found elevated creatine levels in liver and muscle. Conversion of kidney glycoylamine to liver creatine and its incorporation into skeletal muscle were greatly increased. An increase in both creatine synthesis and turnover of skeletal muscle creatine was indicated during tocopherol deficiency. In similar studies with monkeys, Day *et al.* (220) reported increased incorporation of injected formate-C<sup>14</sup> into nucleic acids in both muscle and bone marrow. Using injections of radioactive methionine, Medovar (221) reported that total proteins in heart muscle were lowered during vitamin E deficiency, indicating a reduction in the renewal of total protein. The concentration of most free amino acids was found by Smith & Nelson (222) to be elevated in the tissue of dystrophic animals with only glycine and serine reduced. With elevated tissue levels of various nitrogenous products of protein catabolism, these materials exceed renal thresholds. Thus, creatinuria is characteristic of vitamin E deficiency, as has been demonstrated recently in humans (223), monkeys (224), and swine (225). Verzar & Huber (226) found that the excretion of injected creatine during vitamin E deficiency was only 40 per cent that for normal animals. On the other hand, Beckman & Buddecke (227) found no aminoaciduria but reported urinary excretion of aldolase and an increase in muscle and serum aldolase activity in vitamin E-deficient animals. In human muscular dystrophy, Blahd *et al.* (228) found an increase in the number of urinary amino acids as well as a number of unidentified ninhydrin-reacting constituents. The urine of mothers and siblings of patients with muscular dystrophy also showed a similar increase, suggesting maternal transmission of an inherited metabolic abnormality. Fink *et al.* (229) reported one of the unknown urinary constituents to be 1-methylhistidine. Livers from deficient animals were more active in converting thymine-2-C<sup>14</sup> to 5-hydroxymethyluracil and other intermediates.



*Antioxidant relationships.*—The antioxidant activities of the tocopherols are not related to their biological potencies. In a methyl linoleate system,  $\gamma$ - and  $\delta$ -tocopherols were the most effective and  $\alpha$ -tocopherol the least [Ward & Lee (230)]. As antioxidants, the tocopherols are modified by other substances; for example, amino acids were synergistic with vitamin E in an antioxidant system in herring (231).

Dam (232) comprehensively reviewed the influence of antioxidants on vitamin E deficiency. Two chemical antioxidants, diphenyl-*p*-phenylenediamine (DPPD) and methylene blue, were reported to substitute for tocopherol in preventing either gestation-resorption in rats or encephalomalacia in chicks. Ames *et al.* (233) found both methylene blue and DPPD to be somewhat active in the rat bioassay, which was attributed to a sparing effect on small residual quantities of vitamin E in the diet or body tissues. Draper *et al.* (234) concurred on the low activity of DPPD in vitamin E deficiency but proposed that the dietary requirement of the rat for vitamin E may be reduced when an effective antioxidant is supplied simultaneously. Draper & Johnson (235) supported this hypothesis in reporting that DPPD prevented the development of vitamin E deficiency in lambs fed a tocopherol-free artificial milk diet. This concept was not confirmed by Shull *et al.* (236), who demonstrated that the addition of DPPD or Santoquin (6-ethoxy-1, 2-dihydro-2, 4-trimethylquinoline) delayed but did not prevent the onset of dystrophy in guinea pigs fed vitamin E-deficient diets. Practical applications of the sparing effects of chemical antioxidants should be considered with caution, since DPPD and methylene blue are both toxic, whereas BHT (2,6-di-*tert*-butyl-*p*-cresol) and hydroquinone are not. For example, DPPD prolonged the gestation period of rats, and induced both maternal and infant mortality (233). In general, if the antioxidant is not absorbed as such, it can have little or no sparing effect on the vitamin E in the body. The contention that chemical antioxidants can actually replace vitamin E in metabolic function has not been demonstrated.

Supplementation with  $\alpha$ -tocopherol tends to stabilize easily oxidized constituents in the body. Della Beffa (237) found that administration of vitamin E to humans or guinea pigs increased the amount of glutathione in the blood. Easily oxidized tissues, such as fat, were shown by Mecchi *et al.* (208, 238) to be more stable following tocopherol supplementation of chickens and turkeys. However, Pedersen *et al.* (239) found no increased keeping quality of butter from cows supplemented with 2 gm. of  $\alpha$ -tocopherol daily. Tocopherols in fat were not all destroyed during cooking and were considered to be excellent carry-through antioxidants (240).

Interrelationships of vitamin E and iron have recently been studied. Greenberg *et al.* (241) noted that the rate of hemoglobin regeneration in iron-fed rats was consistently greater following supplementation with ascorbic acid plus vitamin E. Injection of rats with an iron-dextran complex induced localized changes resembling vitamin E deficiency (242). Vitamin E supplementation may be needed to prevent tocopherol-depletion during therapy with iron salts.

*Interrelationships with "Factor 3" and selenium.*—Dietary liver necrosis in rats can be produced by feeding a vitamin E-deficient diet containing high levels of certain types of yeast, such as *Torula* yeast, as the principal protein source. This condition was prevented with vitamin E, cystine, or a nonfat substance termed "Factor 3." By feeding *Torula* yeast to chicks as the main protein source of a vitamin-E-deficient diet, Scott *et al.* (243) rapidly and consistently produced exudative diathesis and prevented it with either dried brewers' yeast or *d*- $\alpha$ -tocopheryl acetate. In chicks with exudative diathesis, Goldstein & Scott (244) found decreased total serum protein, accompanied by a decrease in the albumin to globulin ratio. The exudate proteins had electrophoretic patterns similar to those of plasma proteins indicating increased capillary permeability. Similar findings in turkey poults on a *Torula* yeast diet were reported by Creech *et al.* (245, 246). These abnormalities in rats, chicks, and poults produced on diets high in *Torula* yeast were erroneously attributed primarily to vitamin E-deficiency.

The situation was clarified when Schwarz & Foltz (247) found that selenium was present in "Factor 3" in a bound form and that 0.04 p.p.m. of sodium selenite in the diet prevented liver necrosis. In a collaborative study Scott *et al.* (248) and Schwarz *et al.* (249) found that concentrates of "Factor 3" and selenium derivatives prevent exudative diathesis in chicks. Simultaneously and independently, Stokstad *et al.* (250, 251) also reported that sodium selenite prevented exudative diathesis in chicks. Thus, it became immediately necessary to ascertain to what extent, if any, selenium could replace vitamin E in other conditions attributed to vitamin E deficiency. Sodium selenite and a "Factor 3" concentrate were devoid of biological activity in the rat gestation-resorption bioassay (203). On a vitamin E-deficient diet sodium selenite at dietary levels up to 10 p.p.m. was without activity in chicks in the prevention of either encephalomalacia or mortality (203). Thus, selenium did not replace vitamin E in either of these classic vitamin E-deficiency conditions. The beneficial effect of supplementation with "Factor 3" or selenium derivatives has been demonstrated only on synthetic diets which contain high levels of either *Torula* yeast or isolated soybean protein and which are simultaneously deficient in vitamin E. Decreased utilization of vitamin E was considered by Bieri *et al.* (252) to be a factor.

Scott *et al.* (253) employed a *Torula* yeast diet in bioassaying various feed ingredients for vitamin E-like activity. The responses observed were greater than could be attributed to the chemically determined tocopherol content, indicating wide distribution of selenium or "Factor 3" or "brewers' yeast factor" in natural products. It will be necessary to re-evaluate carefully studies involving exudative diathesis in chicks, liver necrosis in rats, and associated syndromes produced on diets containing high levels of *Torula* yeast. For example, Rabbi *et al.* (254) reported that rats on necrogenic diets were low in betaine-homocystine transmethylase and observed that vitamin E was less active than cystine in maintaining normal levels of this enzyme. Barness *et al.* (255) found that urinary excretion of several ether-soluble acids

in rats fed necrosis-producing diets was prevented by vitamin E supplementation. It remains to be determined whether these effects are due to vitamin E deficiency, to selenium deficiency, or to simultaneous deficiency of both.

*Deficiency and physiology.*—Vitamin E deficiency in the male animal generally leads to deterioration of the germinal epithelium and atrophy of the testes. In rats, these changes are essentially irreversible, whereas in hamsters Mason & Mauer (256) found slower degeneration which was reversed by supplementation with *d*- $\alpha$ -tocopheryl acetate. Most interestingly,  $\alpha$ -tocopheryl hydroquinone, generally considered to have only anti-dystrophic activity, was as effective as *d*- $\alpha$ -tocopheryl acetate in the repair of germinal epithelium. Darlington & Chassels (257) gave daily doses of 1000 I.U. of *d*- $\alpha$ -tocopheryl succinate to thoroughbred racehorses and claimed that winnings were greater and that breeding records of both stallions and mares were greatly improved; unfortunately, it was not feasible to have matched control groups.

In poultry, vitamin E-low diets lead to encephalomalacia in chicks and to reduction of egg hatchability and increased embryonic abnormalities, particularly in turkeys. Supplementation of a corn-soy ration containing 2.5 per cent dehydrated alfalfa meal and 2 per cent fish liver oil with 20 I.U. *d*- $\alpha$ -tocopheryl acetate/lb. resulted in striking improvements in the hatchability of turkey eggs [Jensen *et al.* (258)]. The requirement of vitamin E for breeding turkeys for optimum hatchability was reported by Jensen *et al.* (259) to be in excess of 13.6 I.U./lb. of diet. Later, Jensen & McGinnis (260) found that hatchability on diets supplemented with 24 I.U. *d*- $\alpha$ -tocopheryl acetate/lb. was 5 per cent greater than at a level of 12 I.U./lb. Ferguson *et al.* (261) also indicated that turkeys on a practical diet need supplementary levels of vitamin E for maximum hatchability. Bryan & Moreng (262) found that supplementation of a practical diet with vitamin E improved both fertility and hatchability. Jensen *et al.* (263) observed that dried brewers' yeast did not improve hatchability of turkey eggs, indicating that the "Factor 3"-selenium complex is inactive in this symptom of vitamin E deficiency. Ferguson *et al.* (264) observed lens abnormalities as well as focal areas of acute encephalomalacia in vitamin E-deficient turkey embryos. Winter (265) suggested that an outbreak of nutritional encephalomalacia in Australia in birds on diets containing both bran and alfalfa was due to deterioration of the diet during storage. Jungherr (266) has summarized the various neuropathologic lesions of nutritional encephalomalacia.

Problems associated with vitamin E deficiency in chicks still remain to be solved. On a casein-gelatin diet containing 16 per cent stripped lard, Ames (267) reported growth depression and rapid appearance of encephalomalacia. On the other hand, Scott *et al.* (268) could not produce encephalomalacia in chicks on a cerelose-casein diet, with 3 per cent added fish liver oil. When degerminated white corn replaced the cerelose, encephalomalacia was produced, but with solvent-extracted degerminated white corn, no symptoms were observed (269). Unidentified factors are probably involved in the pro-

duction or prevention of vitamin E-deficiency symptoms. Machlin & Shal-kop (270) observed a muscular degeneration resembling white muscle disease in chicks fed casein-gelatin diets plus 4 per cent cottonseed oil, and prevented the syndrome with  $\alpha$ -tocopheryl acetate. Methionine or cystine likewise prevented this muscular degeneration, but brewers' yeast did not, suggesting that the "Factor 3"-selenium complex was not involved.

Muscular dystrophy in mammals is characteristic of induced vitamin E deficiency. Pappenheimer *et al.* (271) produced dystrophy in guinea pigs and cured it with  $\alpha$ -tocopherol, but a lowered incidence of cures was observed in subsequent deficiencies with the same animals. Rats on a daily intake of about 0.7 mg. of  $\alpha$ -tocopherol showed symptoms of muscular dystrophy at 700 to 900 days of age, suggesting that the vitamin E requirement for maintaining the integrity of striated muscle may increase with age [Berg (272)]. Muscular dystrophy in New Zealand livestock has been reviewed by Hartley & Dodd (273). Myopathy in mature sheep, described by Bosanquet *et al.* (274) was considered by Blaxter & MacDonald (275) to be physiologically similar to enzoötic muscular dystrophy in cattle.

Emmel (276) described a renal manifestation of vitamin E deficiency in rats fed diets containing a high level of unsaturated fatty acids. This abnormality was prevented by *d*- $\alpha$ -tocopheryl acetate, but once established was not readily reversed by tocopherol therapy.

Production of vitamin E deficiency in primates was reported by Dinning & Day (224). Vitamin E-deficiency symptoms in the monkey included muscular dystrophy, creatinuria, allantoinuria, aminoaciduria, a decreased excretion of creatinine, anemia, and granulocytosis. These deficiency symptoms were reversed by treatment with  $\alpha$ -tocopherol. Since anemia was the first sign of vitamin E deficiency observed, they suggest that some cases of unexplained anemia in humans may be the result of tocopherol deficiency. Evidence of vitamin E deficiency in man was presented by Woodruff (277). A patient with xanthomatosis and biliary cirrhosis showed absence of serum tocopherols, increased creatinuria, and urinary excretion of a pentose complex. Tocopherol supplementation resulted in the disappearance of the creatinuria and pentosuria and eventually increased serum tocopherols. These characteristic symptoms of vitamin E deficiency reappeared on administration of placebos. Similarly, Nitowsky *et al.* (278) reporting on infants and children with steatorrhea, found low serum tocopherol levels and decreased resistance to hemolysis of red cells. These symptoms were likewise reversed by vitamin E therapy. Examination of patients with other malfunctions of fat absorption may lead to further knowledge of fat-soluble vitamin deficiencies in man.

#### VITAMIN K

*Chemistry and metabolism.*—Procedures for the purification of 2-C<sup>14</sup>-methyl-3-phytyl-1,4-naphthoquinone (vitamin K<sub>1</sub>-C<sup>14</sup>) and 2-C<sup>14</sup>-methyl-1,4-naphthoquinone (menadione-C<sup>14</sup>, vitamin K<sub>3</sub>-C<sup>14</sup>) were described by

Woods and Taylor (279), who noted appreciable self-decomposition of both labeled vitamins on storage, presumably due to their radioactivity. Richardson *et al.* (280) also noted destruction of vitamin K compounds following irradiation. Synthesis of tritium-labeled menadione was described by Marrian (281).

The spectrophotometric method for the estimation of menadione as the 2,4-dinitrophenylhydrazone was modified [Sathe *et al.* (284)]. The polarographic determination of menadione was described by Jongkind *et al.* (285) and Kékedy & Hajdu (286).

Biosynthesis of menadione by *Aspergillus flavus* was not obtained unless trace amounts of the vitamin were added to the medium (282). Perdue *et al.* (283) reported that menadione sodium bisulfite was 1.5 times as active as vitamin K<sub>1</sub> in maintaining chick-prothrombin levels.

Vitamin K<sub>1</sub>-C<sup>14</sup> was administered by various routes to rats by Taylor *et al.* (287). The largest amount of radioactivity was present in the liver and the levels in the various tissues examined were proportional to the amount of vitamin K<sub>1</sub>-C<sup>14</sup> administered. There was no evidence of metabolism of the 2-methyl group to CO<sub>2</sub>. In further work (288) vitamin K<sub>1</sub>-C<sup>14</sup> was concentrated in the liver of the rat, but menadione-C<sup>14</sup> was not. In pregnant rats both vitamin K<sub>1</sub>-C<sup>14</sup> and menadione-C<sup>14</sup> were found in fetal tissue, indicating that both compounds passed the placental barrier. Chicks fed menadione showed no evidence of conversion to, or liver storage of, vitamin K<sub>1</sub> [Nelson & Norris (289)].

*Enzyme relationships.*—The vitamins K, especially menadione, have been postulated to be components of electron-transport systems. Oxidation of glucose-6-phosphate by a yeast hydrolysate as well as of lactate and succinate by a heart enzyme was accelerated by menadione (290). In studies both *in vivo* and *in vitro* on mitochondria, menadione did not affect oxidation of  $\beta$ -hydroxybutyrate but phosphorylation was inhibited [Schulz & Goss (291)]. It was concluded that menadione functions as a nonphosphorylative shunt in electron transport. Reduced menadione can be enzymatically oxidized by a horse heart muscle preparation in the absence of cytochrome-*c* [Colpa-Boonstra & Slater (292)]. Using the supernatant from *Mycobacterium phlei* cells, Weber & Brodie (293) found a "menadione-dependent cytochrome-*c* reductase" requiring reduced diphosphopyridine nucleotide as an electron donor. Brodie (294) reported inactivation of the enzyme at 3600 Å which also destroys vitamin K<sub>1</sub>, and the presence of a lipid component chromatographically similar to vitamin K<sub>1</sub>. However, the presence of vitamin K in enzyme preparations has not been conclusively demonstrated nor has vitamin K been shown to undergo oxidation and reduction during operation of an enzyme system. Available data suggest participation of vitamin K in electron transport but they are not conclusive.

The role of vitamin K<sub>1</sub> in oxidative phosphorylation has been further investigated. On the basis of increases in P/O ratios following vitamin K<sub>1</sub> addition to vitamin K-deficient chick liver mitochondria, Martius (295) in-

dicated that one of the three phosphorylations accompanying the oxidation of  $\beta$ -hydroxybutyrate involved vitamin  $K_1$ . Dallam & Anderson (296) re-investigated the problem, using rat liver mitochondria treated with ultraviolet radiation, and while disagreeing with Martius' calculations, came to the same conclusion. Brodie *et al.* (297, 294) also found that vitamin  $K_1$  restored oxidative phosphorylation in light-inactivated preparations. Their bacterial system exhibited specific dependence on vitamin  $K_1$  for coupled oxidative phosphorylation. Lapachol methyl ester and vitamin  $K_1$  diacetate showed some activity, whereas menadione and several other compounds with structural similarity were inactive. The reactivated system was uncoupled by dinitrophenol and by dicumarol. Green *et al.* (298) reported that depression of oxidative phosphorylation with dicumarol was not prevented by prior addition of vitamin  $K_1$  and that many other enzymes were unaffected by dicumarol. Thus, oxidative phosphorylation *in vitro* can be enhanced by vitamin  $K_1$  and uncoupled by dicumarol, but the position of vitamin  $K_1$  in the intact phosphorylation chain needs clarification.

*Metabolic effects.*—Hypoprothrombinemia can readily be produced by administration of drugs of the sulfa or dicumarol types and alleviated by vitamin K. Capillary strength in rats was also reduced on feeding dicumarol and increased following vitamin K therapy (299). Both vitamin  $K_1$  and the tetrasodium salt of 2-methyl-1,4-naphthohydroquinone-1,4-diphosphate (Synkavite) were effective in preventing hypoprothrombinemia induced in rabbits by feeding 3-( $\alpha$ -*p*-chlorophenyl- $\beta$ -acetylethyl)-4-hydroxycoumarin (300). Emulsions of vitamin  $K_1$  and aqueous solutions of Synkavite were most effective when administered intravenously (301). If the ratio of vitamin  $K_1$  to dicumarol is held constant, and both are administered simultaneously, hypoprothrombinemia reaches a maximum as the dosage is increased, indicating that vitamin  $K_1$ -dicumarol antagonism is not a simple metabolite-anti-metabolite relationship (302). Prothrombin and plasma thromboplastin component (PTC) [for other synonyms see Blaxter (132)] levels were rapidly depressed in rats fed dicumarol, and both increased to normal within 4 hours following vitamin  $K_1$  administration, indicating a common origin (303). In addition to PTC, plasma thromboplastin antecedent (PTA) levels were lowered in humans with faulty vitamin K absorption or following dicumarol administration, and both were rapidly increased on administration of menadione sodium bisulfite [Naeye (304)]. Chronic lack of bile also induced decreased levels of prothrombin and proconvertin in dogs. This was corrected by administration of vitamins  $K_1$ ,  $K_2$ , menadione, or Synkavite. On oral administration, vitamin  $K_1$  was the least active on a molar basis, whereas intravenously all four had approximately the same molar biological activity [Fisher *et al.* (305)].

In poultry, either sulfaquinoxaline (306, 307) or  $\beta$ -amino-propionitrile (307) in the diet induced a hemorrhagic condition, which was corrected by vitamin K supplementation. The hemorrhage associated with cecal coccidiosis was lessened following supplementation with menadione sodium bisulfite, re-



sulting in decreased mortality (308, 309). The antibiotic activity of menadione bisulfite (310) and Synkavite (311) may explain the resistance to fowl typhoid of chicks on diets supplemented with menadione or menadione bisulfite (312).

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## NUTRITION<sup>1</sup>

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The last few years have seen a growing recognition of the seriousness of protein malnutrition in a large part of the world and a steady increase in the understanding of the scientific bases for the evaluation of protein quality. An indication of this is that investigation into those aspects dealing directly or indirectly with protein metabolism has become a very significant part of the total research in the field of nutritional biochemistry. In view of the large number of articles in this field and the complete coverage given in last year's review to work on lipides, which is another biochemical field of major current nutrition interest, no attempt has been made to deal with articles unrelated to the main topic of protein nutrition. A special effort has been made, however, to give consideration to the reports of workers in technically underdeveloped countries who are dealing with protein malnutrition as an urgent national problem. Unfortunately, many interesting papers on even this more limited subject could not be mentioned in the space allotted.

### PROTEIN REQUIREMENTS

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<sup>1</sup> Based primarily on articles published in 1956 and the first eight months of 1957.

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## NUTRITION<sup>1</sup>

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The last few years have seen a growing recognition of the seriousness of protein malnutrition in a large part of the world and a steady increase in the understanding of the scientific bases for the evaluation of protein quality. An indication of this is that investigation into those aspects dealing directly or indirectly with protein metabolism has become a very significant part of the total research in the field of nutritional biochemistry. In view of the large number of articles in this field and the complete coverage given in last year's review to work on lipides, which is another biochemical field of major current nutrition interest, no attempt has been made to deal with articles unrelated to the main topic of protein nutrition. A special effort has been made, however, to give consideration to the reports of workers in technically underdeveloped countries who are dealing with protein malnutrition as an urgent national problem. Unfortunately, many interesting papers on even this more limited subject could not be mentioned in the space allotted.

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Evidence for lowering recommended allowances for protein has come from various sources. Rose & Wixom (3) concluded that when the eight essential amino acids were administered at the "safe" levels of intake (4), nitrogen equilibrium could be maintained with the addition of nonessential amino acid nitrogen in the form of glycine to provide a total daily intake of only 3.5 gm. of total nitrogen. Calculated as nitrogen  $\times 6.25$ , this is only about 0.32 gm. of protein per kg. body weight for the lightest subject tested and 0.25 for the heaviest one. In 171 apparently well-nourished young women, 17 to 27 years of age, consuming self-selected diets in the United

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States, the average daily intake was  $0.84 \pm 0.19$  gm. of protein per kg. body weight according to five-day balance studies by Scoular *et al.* (5). Barness *et al.* (6) studied nine healthy infants under one month of age fed isocaloric and isonitrogenous formulas of cow's milk and breast milk for alternate periods. Two of these infants remained in nitrogen balance and grew satisfactorily on 0.8 gm. protein per kg. body weight per day on either formula.

Persons consuming poor vegetarian diets can attain nitrogen balance at low intake levels, but at the cost of adequate nitrogen stores or poor growth or both. For example, Murthy *et al.* (7) have reported that, even on a rice diet, five girls aged seven to nine were in nitrogen balance on a total calorie intake of 1010 and a protein intake of 1.2 gm. per kg. body weight per day. Sur *et al.* (8) studied 12 children, eight to eleven years of age, maintained on either a rice diet or a rice-peanut curd diet providing mean daily protein intakes of approximately 1.1 and 1.5 gm. per kg. body weight respectively. Despite the low protein intake, only one of the children (on the rice diet) showed a negative nitrogen balance. Subrahmanyam *et al.* (9) also report adults in nitrogen balance on 1.2 gm. of protein per kg. body weight when eating a vegetarian diet with an apparent protein digestibility of only 50 per cent.

Recent reports on the poorer nitrogen utilization during caloric restriction are based on work with rats (10 to 13), mice (14) and poultry (15, 16). Any decrease in the apparent digestible energy of the diet is compensated for by increased food consumption (13, 17). Sibbald *et al.* (17) found that the ratio of apparent digestible energy to apparent digestible nitrogen of the food controlled the percentage of nitrogen retained. The optimum ratios appeared to be 250 to 300 calories of apparent digestible energy per gm. of apparent digestible nitrogen. In a more recent article, the same authors (18) confirmed these findings and showed that they apply also to a protein-free amino acid diet.

Evidence that dietary fat has a specific protein sparing action independent of its caloric value, comes from work with rats [Cohn & Joseph (19); Schreiber & Elvehjem (20)] and dogs [Ontko, Wuthier & Phillips (21)]. Evidence to the contrary, however, is exemplified by the recent work of Metta & Mitchell (22) who found that, when fed in isocaloric amounts, dietary fat and carbohydrate have essentially the same effect on protein utilization.

Heinicke, Harper & Elvehjem (23), working with guinea pigs, found no differences in protein requirements between diets containing dextrin or sucrose. Womak & Marshall (24), however, report that in experiments on rats with low levels of amino acid intake both sucrose and fructose resulted in greater negative nitrogen balance than starches or corn dextrin. The data of Cornely, Barness & György (25) also suggested that nitrogen retention was greater in lactose-fed babies than in those for whom dextrin-maltose was the carbohydrate source. That the effect of certain carbohydrates on the over-all nitrogen need of the animal could be due to interference with digestibility has been shown by Yoshida & Morimoto (26) who found marked de-

creases in casein digestibility in rats when raw (but not steamed) potato starch was included in the diet. The effect of the type of carbohydrate on amino acid requirements has been well reviewed by Harper & Elvehjem (27).

It has long been recognized that urea can fill part of the nitrogen needs of ruminants, but it is now clear that this substance can also be used by rats [Rose & Dekker (28)], hamsters [Matsumoto (29)], human adults (3) and even premature infants [Holt & Snyderman (30)]. It is noteworthy that breast milk contains approximately 20 per cent of its nitrogen in the form of urea and other nonprotein compounds, and cow's milk only about 5 per cent. Despite this the nitrogen retention calculated as percentage of total nitrogen intake has been shown to be slightly (but not significantly) higher in breast milk (6). Although Finlayson & Baumann (31) report that both diammonium citrate and urea exert a growth depressing action in rats in either spaced or *ad libitum* feeding, the levels employed were extremely high.

Since the nitrogen balance method is used by most investigators studying protein and amino acid requirements in humans, the critique of this technique by Lowe & Pessin (32) is useful. In addition, Allison & Wannemacher (33) stressed that the requirement for maintaining adequate nitrogen reserves is greater than that for nitrogen balance. Meyer (34) showed that each added increment of cellulose increased the metabolic fecal nitrogen, although there was no influence of fiber on urinary endogenous nitrogen in growing rats. This emphasized again the importance of maintaining constant the fiber content of experimental diets in balance studies of the digestibility and biological value of proteins.

#### BIOLOGICAL VALUE OF FOODSTUFFS AND DIETS

*Assay methods.*—More attention than ever has been centered on evaluating the biological value of plant and animal proteins, although the methods used have for the most part been well established. Bender (35) has shown that the Protein Efficiency Ratio as determined in rats varies directly with food intake and does not represent true nitrogen utilization since total body weight used for the calculation of the Protein Efficiency Ratio is affected by other factors such as fat deposition. Bender & Doell (36) have advocated a shorter method of assessing protein quality by feeding rats at a 10 per cent level of test protein and another group of litter mates a protein-free ration. The algebraic difference between the gains in weight of the two groups divided by the weight of protein eaten at the end of ten days is called the Net Protein Ratio. The Net Protein Ratio can be converted to a percentage scale by multiplying by the factor 16, to give the Protein Retention Efficiency.

Miller & Bender (37), Bender & Doell (38) and Dreyer (39) described the calculation of the Net Protein Utilization by the formula  $(B_F - B_K + I_K) + I_F$ , where  $B_F$  is the carcass nitrogen of protein-fed rats,  $B_K$  the carcass nitrogen of rats on a protein-free diet,  $I_F$  total dietary nitrogen and  $I_K$  nitrogen from other than the test protein. They believe the Net Protein Utilization



to be a more precise estimate of the proportion of dietary nitrogen retained by the carcass of the animal. Net Protein Utilization, as determined by carcass analysis, correlates highly with the Protein Retention Efficiency, but the latter has the advantage of combining both maintenance and growth requirements for protein.

Chick assays have been used for many years for the evaluation of the biological value of proteins, but Beaty *et al.* (40) have advocated the use of chicks which have been protein-depleted to about 75 per cent of their initial weight. They assay the protein under test at dietary levels of 6.5, 9 and 12 per cent protein. The protein depletion-repletion technique is a sensitive measure of protein quality in baby pigs according to Peo *et al.* (41). The same authors (42) emphasize that as levels of dietary protein increase, the differences in protein quality of the test foods become less critical. Meade (43) and Meade & Teter (44) have also stressed that the relative proportion of amino acids is more important when the total amount of dietary protein is inadequate.

These observations, which are of great practical importance, are well supported by a study by DeMaeyer & Vanderborcht (45) which shows that the biological value of soya fed to children, as determined by nitrogen retention, is higher at high levels of intake than at low. This is also suggested by Sure (46) who has studied the nutritive value of the proteins in various foods at increasing protein intakes and found 15 per cent to be the most efficient level for poultry and livestock feeding. However, human nutrition is concerned with the end result as well as efficiency, and increasing the amount fed of a protein of intermediate value may be as nutritionally effective as improving its quality.

Allison (47) has continued to call attention to the fact that nitrogen balance is only the sum of the over-all nitrogen metabolism and does not measure the gain or loss of protein from specific organs or tissues. Recent work by Vijayaraghavan (48) gives further evidence that growth and nitrogen balance are not the only criteria by which the nutritive value of protein should be judged. In his study casein, egg, meat, wheat gluten, and rice protein varied widely in their efficiency for blood formation in rats. Perdue, Lambert & Frost (49) show human and dog blood to be poor sources of essential amino acid nitrogen for protein-depleted rats. This emphasizes the well-known fact that special tissues may have amino acid requirements different from those for growth and maintenance.

Sheffner *et al.* (50, 51) have used the microbiological determination of the pattern of amino acids released *in vitro* by pepsin to reveal differences between proteins which were not apparent from their total content of each essential amino acid. They have proposed a new index, the Pepsin Digest Residue, which, when divided by the digestibility, accurately predicted the biological value of such diverse products as egg, soya, casein, yeast and white flour. Additional microbiological methods for measuring the utilization of intact protein have been advocated by Mertz *et al.* (52) using *Pseudomonas*

*aeruginosa*, by Fernell & Rosen (53, 54) employing *Tetrahymena pyriformis* and by Teeri, Virchow & Loughlin (55) using enzymatic hydrolysis of the sample prior to assay with *Streptococcus fecalis*.

*Amino acid reference pattern.*—The review of Flodin (56) documents well the principle that the amino acid pattern found in animal proteins provides superior efficiency of protein utilization for periods of high anabolic activity and for the maintenance of labile protein stores. It also closely parallels the pattern of amino acids found best for rat growth and for maintenance of nitrogen balance in adults. For example, Albanese *et al.* (57) have provided further evidence that the utilization of dietary proteins increases as their proportion of lysine and tryptophan approaches that of muscle tissue. For evaluating protein-containing foods, they propose the calculation of a Protein Utilization Index which is equivalent to the body weight gain  $\times$  the nitrogen retention in mg. per kg. body weight  $\div 100$ .

These considerations led the F.A.O. Study Group (2) to designate a theoretical reference protein, the amino acid pattern of which could be used for calculating a "protein score" based on the per cent deficiency of the most limiting amino acid. The F.A.O. reference amino acid pattern in gm. of amino acid per gm. of nitrogen is as follows: isoleucine, 270; leucine, 306; lysine, 270; methionine and cystine, 90; phenylalanine and tyrosine, 180; threonine, 180; tryptophan, 90; and valine, 360. Preliminary evidence obtained by the authors (58) suggests that the pattern may be too low in lysine and too high in methionine. The principle of such a reference pattern is very useful, and the necessary research to perfect it should have a high priority.

*Amino acid availability.*—Deshpande *et al.* (59) have taken advantage of the low isoleucine content of blood meal to demonstrate the availability of isoleucine in eight different proteins used to supplement it. They concluded that isoleucine is available for the rat only to 30 per cent in zein, 60 to 70 per cent in casein and gelatin, and over 90 per cent in beef fibrin, egg albumin, and Drackett (soybean) protein. Gupta & Elvehjem (60), using rats, found that the biological availability of tryptophan from beef and pork protein, egg albumin, beef fibrin, purified soybean protein, and milk powder varied between 80 and 100 per cent. Stevens & Henderson (61) obtained similar results for casein. Kratzer & Green (62) found that the availability of lysine in spray-dried soluble blood meal for the chick was only 75 per cent of that found by microbiological assay after acid hydrolysis. Vat-dried blood meal had available only 60 per cent of the lysine determined microbiologically. The many studies of amino acid destruction with food processing and storage are beyond the scope of this review.

#### IMPROVEMENT OF PROTEINS

*Vegetable mixtures and mixed diets.*—An excellent report from the Indian Council of Medical Research (63) reviewed work in China and India on the preparation, testing and use of milk substitutes for areas where milk production is inadequate. The report concluded that milks made from soybeans,

peanuts, cashew nuts, coconuts, almonds, or legumes can be helpful in supplementing poor cereal diets. In the Belgian Congo a milk, prepared from peanuts, given to 96 lactating women and 135 infants by Holemans *et al.* (64) proved to be a good supplement as judged by biochemical and clinical indices.

A report from Russia (65) described several mixtures of cereals and legumes which proved to have a high nutritive value, the best of which was better than casein in rat growth tests, and contained 60 per cent buckwheat, 20 soya and 16 rice. Scrimshaw *et al.* (58) have described a 25 per cent protein mixture for human feeding which contains 50 per cent dried corn "masa" (from lime-treated corn), 35 per cent sesame oil meal, 9 per cent cottonseed meal, 3 per cent kikuyu leaf meal and 3 per cent torula yeast. This mixture was as effective as cow's milk when tested isonitrogenously and isocalorically in rats, dogs, and well children.

Ladell & Phillips (66), in human nitrogen retention studies, found that, provided there is a certain minimum amount of animal protein (at least 14 gm.), one part of animal protein may be replaced by 2.2 parts of peanut protein. Desikachar, Sankaran & Subrahmanyam (67) and Phansalkar & Patwardhan (68) have used rats to demonstrate the improvement in nutritive value obtained in cereal proteins by adding legumes. The paper of Kamath & Sohoni (69) was one of the latest to show that the amino acid content of green leaves, while inferior to casein, is a potentially useful supplement to cereal diets. Other potential sources of vegetable protein which rat growth trials have shown capable of supplementing cereal diets are the green algae, *Scenedesmus obliquus* and *Chlorella pyrenoidosa* [Hundley, Ing & Krauss (70)], the South American Quinoa, *Chenopodium quinoa* [Quirós-Pérez & Elvehjem (71)] and Buckwheat, *Fagopyrum tartaricum* [Sure (72)].

The many recent studies on the nutritive value of fish and fish products cannot be referred to individually, but reports have also appeared on the use of fish products as supplements to cereals. Costamailere & Ballester (73) showed that fish flour was as effective as whole milk powder in improving the rate of growth of rats on a diet patterned after that consumed by low economic groups in Chile. Cravioto *et al.* (74) found that five of seven fish flours added at a level of 10 per cent of a "tortilla" diet, improved the biological value of the corn protein as indicated by rat growth experiment. The two fish flours which were ineffective had been deodorized by industrial processing. Sure (75) and Carpenter *et al.* (76) have found that fish flours make effective supplements to cereal diets.

**Corn.**—In view of the poor biological value of the protein of corn, it is surprising that the only published reports of improving the biological value of predominantly corn diets by use of complementary vegetable proteins were those of Scrimshaw *et al.* (58) previously referred to, and Mangay, Pearson & Darby (77) who showed that millet, *Setaria italica*, will correct the niacin deficiency induced in rats by a 9 per cent casein and 40 per cent corn diet. Mangay *et al.* (77) also showed that 1 per cent lysine added to a diet of 40 per cent maize and 40 per cent millet improved growth consider-

ably, but produced no growth response when added to a diet of 80 per cent maize and 10 per cent millet unless niacin or tryptophan or both were also added. It is thus clear that millet improves the tryptophan but not the lysine deficiency of corn. Scrimshaw *et al.* (58) have shown some improvement in nitrogen retention of young children when tryptophan was added to a corn "masa" plus gluten mixture but a much greater increase when both tryptophan and lysine were supplied to the levels of the F.A.O. amino acid reference pattern. For rat growth, Hogan *et al.* (78) have reported lysine to be the most limiting amino acid in whole corn, with tryptophan second. Mosqueda-Suarez (79) found that the addition of 0.2 per cent tryptophan to a degermed corn product increased the rate of weight gain of rats 214 per cent, addition of lysine alone, 59 per cent and supplementation with both amino acids 414 per cent.

*Wheat.*—Many studies have shown that supplementing wheat products with lysine results in an improvement of their protein quality. Among the most recent are those of Hutchinson, Moran & Pace (80, 81); Sure (82); Deshpande, Harper & Elvehjem (83); and Rosenberg (84). Deshpande *et al.* (83) showed that lysine prevented fatty livers which developed on a low-protein diet (5.4 per cent) in which wheat was the principal protein source.

It has also been shown that milk and meat can supplement a wheat flour basal diet and make lysine supplementation unnecessary [Westerman, Hays & Schoneweis (85); Westerman, Kannarr & Rohrbough (86)], although Jahnke & Schuck (87) demonstrated that lysine addition is beneficial even when the wheat flour was already enriched with three per cent nonfat milk solids. Sarett (88) studied a mixture of 70 per cent of a cereal food (Pabulum, Mead Johnson & Co.) and 30 per cent milk powder and found that as measured by rat growth it was not improved by lysine supplementation, a result which was to be expected since the basal diet contained adequate lysine to satisfy the rat requirement.

*Other vegetable protein sources.*—The supplementation of other vegetable proteins with synthetic amino acids has also received attention. The work on beneficial effects of the supplementation of most animal feeds with methionine or lysine or both has been well reviewed by Rosenberg (84). Cowlishaw *et al.* (89, 90) found that leaf protein concentrate for children was improved by lysine but not by methionine addition. Kik (91, 92) has demonstrated that the proteins of commercial rice bran and rice polishings, as the sole source of protein in rats, can be improved by the addition of 0.2 per cent L-lysine and 0.2 per cent DL-threonine. The addition by Sure (93) of 0.4 per cent L-lysine, 0.5 per cent DL-threonine and 0.5 per cent DL-methionine to the proteins in milled barley resulted in 151 per cent increase in growth and 224 per cent increase in the Protein Efficiency Ratio. Supplementation of peanut flour with 0.5 per cent each of DL-methionine and DL-threonine improved weight gain 60.6 per cent and Protein Efficiency Ratio 61.5 per cent.

Obviously, foods are not improved by amino acid addition unless a limiting deficiency is corrected. For example, lysine addition failed to im-

prove soybean protein [Block *et al.* (94)]. That more than one essential amino acid may be necessary to bring about satisfactory improvement in a cereal protein is illustrated by the studies of Sure (95) on rice and wheat, and Hundley *et al.* (96), Kik (91, 92), and Deshpande *et al.* (97) on the supplementation of rice.

#### AMINO ACID REQUIREMENTS

*In man.*—The F.A.O. Committee on protein requirements (2) took the important step of considering amino acid as well as protein requirements for man. Basic to the discussion was a series of papers by Rose and co-workers, published from 1947–55, describing the minimal amounts of individual essential amino acids required to maintain “a distinct positive balance, as measured by the average of a period of several days.” These reports were well summarized in an article by Elvehjem (98) and in a comprehensive and critical review by Rose (4).

Available to the F.A.O. Committee, although still unpublished at that time, were a similar series of studies by Leverton *et al.* carried out on young women. In these studies the requirement range reported for threonine was found to be 103 to 305 mg. per day (99); for valine 465 to 650 mg. (100); for tryptophan 82 to 157 mg. (101) and for leucine 170 to 620 mg. (102). Phenylalanine was studied with diets furnishing 900 mg. of tyrosine daily, and, under these circumstances, 120 to 200 mg. were required (103). Swendseid, Williams & Dunn (104) in similar studies on young women found a range of 350 to 550 mg. for methionine plus cystine, and Swendseid & Dunn (105) have reported a range of 250 to 450 mg. for the isoleucine requirement.

All of these figures tend to be lower than those found previously for men, but as Rose points out (4), they are based on a different criterion for nitrogen equilibrium, namely “The zone in which the difference between the intake and excretion does not exceed 5 per cent.” Thus, eight of the sixteen subjects reported to be in nitrogen equilibrium by Leverton *et al.* (99) in the threonine study would have been considered in negative balance by Rose. Another difference, possibly contributing to the lower values for women, was the practice of adjusting the calories to prevent gain or loss of body weight instead of keeping them constant throughout an experiment as done by Rose.

Jones, Baumann & Reynolds (106) have studied the lysine requirement of women and reported it to vary from 400 to 500 mg. per day. These investigations used diets providing all or almost all of the dietary nitrogen as synthetic amino acids and urea or ammonium citrate. Doubt remains as to the errors introduced by determining amino acid requirements on diets containing only minimal quantities of other essential amino acids. Nasset (107) showed clearly that for rats a mixture based on the mean minimum requirements of the nine essential amino acids is grossly inferior to either egg protein or an amino acid mixture which simulates egg protein. In the first of a projected series of papers on the amino acid requirements of both men and

women, Clark *et al.* (108) used cereals and other foods to provide about half of the total nitrogen. They report that ten men and women maintained nitrogen equilibrium at 500 to 900 mg. of lysine. No sex differences were apparent. Since both of these studies employed criteria for nitrogen equilibrium similar to those of Rose, it was reassuring that their results agreed well whether the diet was entirely or only partially synthetic.

As pointed out in the previous section on protein requirements, it must not be assumed that measuring the requirements for nitrogen balance necessarily gives the requirements for optimum nutrition. Schultze (109) found mixtures of amino acids that supported "normal" reproductive and lactation performances in rats for as long as four successive generations and still were not completely adequate for optimum pre- and postweaning weight gain of the young nor for the prevention of fatty livers in the mothers during lactation. The work of Allison *et al.* (110) illustrates the point that the nutritional demands of tissues of an animal may sometimes be revealed more adequately by studying the effects of various stresses upon nutrient requirements.

Snyderman *et al.* (111) have restudied the lysine requirement of six normal infants using a synthetic diet made up of 18 L-amino acids in the same proportion found in breast milk; all six required less than 90 mg. of lysine per kg. body weight per day. The information available on phenylalanine and threonine requirements in children is well summarized by Holt & Snyderman (30).

*In rodents.*—Additional studies of amino acid requirements of rats have also appeared. Forbes, Vaughan & Norton (112) have employed a measure which they refer to as "minimum nitrogen loss" (exogenous urinary nitrogen ÷ nitrogen intake) × 100. The least amount of isoleucine required for minimum wastage of dietary nitrogen in growing rats fed synthetic diets was 2.6 per cent of the conventional protein equivalent of the diets regardless of its total nitrogen content. The requirement of the adult rat for histidine was re-investigated by Moore & Wilson (113) and this amino acid found to be essential for normal nitrogen retention. The discrepancy between this conclusion and most earlier reports may be due to differences in the composition of the experimental diet. Hartsook & Mitchell (114) have demonstrated that the percentage of methionine plus cystine required in the diet of growing rats decreases in an exponential manner with age, while it increases as percentage of the protein requirement from 7 to 8 per cent, probably due to the continued synthesis of keratin for hair, despite the decreasing rate of body growth with age. Womack, Snyder & Rose (115) showed that D-valine is relatively ineffective for promoting rat growth even at the 2 per cent level and still less utilized when L-leucine in the diet is replaced by twice the amount of DL-leucine. Apparently, D-valine is difficult for the organism to use under any circumstance and more so when other D-amino acids are fed.

The second in a series of papers by Heinicke, Harper & Elvehjem (116) has also appeared showing that guinea pigs have a particularly high need for



arginine and methionine which decreases as they mature, paralleling the decrease in growth rate.

*In the chick.*—Baldini & Rosenberg (117) have found the methionine requirement of the chick to increase as the productive energy level of the diet increases. Relating the requirement of a nutrient to the productive energy level of the diet is applicable to most of the nutrient requirements for the chick. For example, Griminger, Scott & Forbes (118) have shown that as the dietary bulk increases the chick requirement for tryptophan and arginine, as percentage of the diet, decreases. Williams & Grau (119) have similarly observed that when 12 per cent or more of cellu-flour replaced glucose, thus reducing the digestible energy concentration of the diet, feed consumption and growth increased in all diets deficient in lysine. When sufficient energy is available, the requirement of the broiler for methionine increases as the protein content of the diet increases [Rosenberg & Baldini (120)]. Griminger, Scott & Forbes (121) have shown that the tryptophan requirement of the growing chick increases with the protein level, although at a slower rate than the latter.

Fisher (122) has shown that, in the presence of sufficient tyrosine, 0.5 per cent of L-phenylalanine was required for chick growth and maximum feed utilization. Since previous studies have established a requirement of 0.9 per cent of the DL-form, it appears that chicks, like rats, do not utilize D-phenylalanine efficiently. The L-tyrosine requirement in the presence of 0.46 per cent L-phenylalanine has been reported by Fisher, Johnson & Leveille (123) to lie between 0.3 and 0.5 per cent.

#### AMINO ACID INTERRELATIONSHIPS

The authors regret the impossibility of including, within the space of this review, the many valuable studies of the metabolic relationships between amino acids and other metabolites. It is essential, however, to refer to the important recent studies of amino acid interrelationships.

Since Elvehjem has been directly involved in much of this work, his review articles are particularly informative (124, 125, 126). He distinguishes between amino acid imbalances, antagonisms between two amino acids and toxic effects of excessive amino acid levels. Benton *et al.* (127) have shown antagonism between isoleucine and valine, between phenylalanine and isoleucine, and between phenylalanine and valine in rats fed a 9 per cent casein diet supplemented with various amino acids. In a subsequent article these authors (128) showed that threonine prevents the growth depression caused by 3 per cent DL-phenylalanine or 3 per cent tyrosine under the above conditions.

Rerat, Bouffault & Jacquot (129) have shown that lysine can produce growth retardation in rats by either a deficiency or an excess in a diet based on corn or wheat gluteins. Similar systematic studies are needed for each of the other essential amino acids. Wu & Lewis (130) found that a deficiency of lysine or of lysine and tryptophan greatly enhances the excretion of



homogentisic acid following the injection of DL-phenylalanine. This effect is abolished by supplementing the gliadin diet with lysine but only partially abolished by supplementing zein with lysine and tryptophan.

The work of Sauberlich (131), while entitled "Amino Acid Imbalance as Related to Methionine, Isoleucine, Threonine and Tryptophan," only shows again that the growth of rats is decreased and food consumption per gm. of gain in body weight is increased when any of these amino acids is deficient and that the restoration of the deficient amino acid corrects these changes.

Gessert & Phillips (132) have reported that either methionine or lysine alone added to a mixed diet for growing dogs caused a growth depression which could be overcome by adding both together. The observation is of qualitative interest even though the use of literature values for the amino acid content of the diet ingredients precludes quantitative conclusions.

#### PROTEIN AND AMINO ACID METABOLISM

*Protein turnover.*—A number of studies not directly related to problems of deficiency have contributed to knowledge of protein and amino acid metabolism. The urinary excretion of  $I^{131}$  after administration of labeled albumin has provided a direct measure of albumin degradation. In four of a group of five human adults in good nutritional state studied by Fremont-Smith & Iber (133) a sudden increase in nitrogen intake from 0.5 to 3.0 gm. per kilo body weight was associated with a slight but definite increase in the albumin degradation rate which persisted even after nitrogen equilibrium was restored. Nitrogen<sup>15</sup> tagged yeast protein has been used by Sharp *et al.* (134) to show that neither achlorhydria nor age in adults depresses the capacity to absorb labeled protein during high protein intake. Sharp *et al.* (135), using the same technique, found the average retention of two younger subjects (age 24) to be greater than that of four older ones (age 51 to 66), the values being 57.6 per cent and 49.1 per cent, respectively. The physiological half life of N<sup>15</sup> from yeast was 61 days in the younger subjects and 86 days in the older. The average total daily requirement for nitrogen from the "pool" (both fed and recycled nitrogen) was found to be 0.23 gm. per kg. body weight.

#### PROTEIN DEFICIENCY IN EXPERIMENTAL ANIMALS

Studies of protein depletion in animals are relatively few compared to the large number of papers which have appeared in the past two years on human protein malnutrition. Stanier (136) and Widdowson & McCance (137) have investigated the composition of muscle, liver, skin, and carcass of adult rats after approximately two months on a diet very low in protein (a) and a diet very high in protein (b) but limited to keep animals at the same weight as with the low protein diet. Diet a was similar to that responsible for kwashiorkor in children and diet b similar to that causing marasmus; changes in liver composition in the rats were very similar to those reported

for each of these two clinical conditions. Iacobellis, Muntwyler & Dodgen (138) found that either protein or potassium depletion resulted in increased lysine and arginine and lowered aspartic and glutamic acids in skeletal muscle, diaphragm, and kidney. Since simultaneous protein and potassium depletion caused no alteration in amino acid pattern, attention was directed to the probable importance of maintaining a correct protein to potassium ratio in the diet therapy of human malnutrition.

Dogs were depleted of body proteins by repeated phlebotomy superimposed on a low protein diet in work reported by Hahn, Baugh & Meng (139). Despite relatively little change in total plasma protein levels, a marked decrease in albumin and increase in alpha, beta, and gamma globulins was observed. Of particular importance is the observation that anaphylactic reactions following immunization to horse serum were diminished or absent in the depleted animals. Of similar interest are papers which suggest mechanisms whereby protein deficiency may reduce resistance to  $\beta$ -haemolytic *streptococci* in rabbits (140), *Salmonella typhi* in rats (141) and tuberculosis in hamsters (142).

Srinivasan & Patwardhan (143) have shown clearly that the growing rat reacts to induced protein deficiency more rapidly and suffers greater histological and biochemical damage than adult animals. Liver xanthine oxidase in mice was shown by Mangoni, Pennetti & Spadoni (144) to decrease with decreasing protein intake within 21 days at dietary levels of 8, 18, and 30 per cent protein. The decrease was accentuated in those groups given 24 mg. of xanthine per 100 gm. body weight. Of fundamental interest is the investigation by Ross & Batt of the relationship between the activity of four hepatic enzymes and diet (145) and age (146). A high casein diet altered the pattern of enzyme activity from that of young to that of old animals and a low casein diet had the opposite effect.

The demonstration by Magee & Hong (147) that 1 per cent of a test amino acid added instead of additional casein to a 7 per cent casein diet can alter pancreatic enzyme activity is pertinent to kwashiorkor studies. Methionine increased lipase and protease activities, phenylalanine or isoleucine increased protease activity alone and no amino acid supplement tested increased amylase. Magee & Anderson (148) found that valine stimulated lipolytic and proteolytic enzyme activity in the pancreas of rats on a similar diet.

While the period was relatively barren of studies of the relationship between protein deficiency and endocrine changes, Gopalan (149) working with monkeys fed diets containing either 20 or 3 per cent protein, has added to his series of excellent contributions on this subject.

#### PROTEIN DEFICIENCY IN MAN

During the review period, at least fifty papers have appeared contributing to the understanding and solution of the problem of severe protein malnutrition in children, now well known by the name kwashiorkor. Most as-

pects of the problem have been reviewed in a series of papers by the senior author and his colleagues. These deal with characteristics (150), response to protein therapy (151), and epidemiology and prevention (152). This information has also been compiled in a monograph (153). Waterlow & Vergara (154) in a publication on protein malnutrition in Brazil and Gopalan & Ramalingaswami (155) in a review of kwashiorkor in India also give authoritative summaries of recent knowledge. An article by Waterlow & Scrimshaw (156) documents the international agreement on the basic features of kwashiorkor and leaves little doubt that they are essentially the same wherever the syndrome is encountered.

In studies of the liver in kwashiorkor, Waterlow (157) has found that, although fatty infiltration and loss of liver protein are both severe in kwashiorkor, the degree of these changes is not a reliable measure of the severity of the case. He also noted that the total nitrogen lost from muscle is much greater than from the liver. Waterlow & Weisz (158) showed that the average loss of liver protein and ribonucleic acid is 40 per cent in Jamaican cases calculated on the assumption that the amount of deoxyribonucleic acid per cell and per liver remains constant even during severe malnutrition. Both Waterlow & Patrick (159) and Burch *et al.* (160) have studied the activity of a number of enzymes in liver biopsy specimens from children with kwashiorkor. Cytochrome-*c* reductase (159, 160) cytochrome oxidase (159), lactic dehydrogenase (159), malic dehydrogenase (159, 160), succinic dehydrogenase (160), glycolic acid oxidase (160), and transaminase (159, 160) were not significantly reduced when calculated per unit of protein. Xanthine oxidase and D-amino acid oxidase (160), however, were highly significantly decreased on both a weight basis and per unit of protein.

Bras, Waterlow & DePass (161) report pancreatic pathology in "malnourished" infants and children and also in marasmic children. Both Waterlow *et al.* (162) and Senecal & Dupin (163) emphasize the necessity of distinguishing between kwashiorkor and marasmus ("Inanition") and point out that in the latter the liver is not fatty. Senecal & Dupin (163) re-emphasize that the fatty liver of kwashiorkor occurs because of an excess of calories relative to protein whether the total calorie intake is high or low.

Badr-El-Din & Aboul-Wafa in Egypt (164) confirmed previous findings of a marked reduction or absence of trypsin activity and the lesser reduction of amylase activity in infants with kwashiorkor. Their observation that trypsin activity is also reduced in marasmus, however, contradicts another recent report (165). Mehta, Venkatachalam & Gopalan (166) showed that children with Nutritional Oedema Syndrome (kwashiorkor) receiving diets containing negligible amounts of fat, excrete more fat in the feces than they consume.

The blood serum biochemical findings in kwashiorkor, which have been summarized in the papers cited previously (150, 153, 154, 155) include serum protein changes; lowered levels of fat soluble vitamins; decreased blood serum activity of cholinesterase, lipase, amylase and alkaline phosphatase;

and lowered blood serum cholesterol and total lipides. Ramanathan (167) calls attention to the lowered blood urea, nonprotein nitrogen, total cholesterol and cholesterol esters as well as total nitrogen, but found the gamma globulin fraction high. The serum electrophoretic patterns reported by Bollo & Montero (168) in Chile and Senecal *et al.* (169) in French West Africa are in agreement with the many previous studies of electrophoretic patterns in this syndrome which show albumin both absolutely and relatively decreased and gamma and alpha globulin fractions relatively increased.

Four technically superior papers on intracellular composition and homeostatic mechanism of infants with severe chronic malnutrition have recently been published by Gómez *et al.* (170 to 173). By analysis of blood serum, muscle, and skin it was found that, independent of decreases or increases in its volume, the extracellular fluid was hypotonic. The intracellular fluid volume was increased except in patients who were classified as "atrophy" (marasmus). Among other electrolyte changes, the potassium content of muscle tissue was found reduced to a larger degree in the patients presenting edema. Unfortunately, the cases varied very greatly in their relative proportions of marasmus and kwashiorkor, and many of them presented secondary complications. Politzer & Wayburne (174) have similarly found no correlation between the degree of edema and either serum  $\text{Na}^+$  or  $\text{K}^+$  levels, nor was the severity of diarrhea correlated with serum  $\text{Na}^+$ . The serum  $\text{K}^+$  levels were reduced only in the subjects suffering from moderate to severe diarrhea.

In five cases of kwashiorkor studied by Ramachandran, Venkatachalam & Gopalan (175), the 24-hour urinary excretion of 17-ketosteroids was much lower than in 12 well-nourished children, but returned quickly to normal with the administration of a high protein diet. Venkatachalam, Srikantia & Gopalan (176) also studied urea space and nitrogen balance during the recovery of eight undernourished adults and found that changes in body weight gave a misleading picture of the actual gain in tissue during recovery due to variations in the water body content.

Cheung *et al.* (177) in three children with kwashiorkor have used column chromatography to study the excretion of amino acids. The distinctive findings were a high ratio of isoleucine to leucine due to an increased excretion of isoleucine and a high phenylalanine to tyrosine ratio due to the increased excretion of phenylalanine. A strikingly low output of threonine was seen in two of the patients. Additional cases must be studied under more standardized conditions before these results can be generalized. Although data of Garrow (178) on the increased  $\text{S}^{35}$  uptake in two Jamaican children with marasmus (one with a large groin abscess) and one with mild atypical kwashiorkor are too preliminary for conclusions regarding either condition, he also shows that dogs depleted of 0, 17.7, 21.1 and 37.7 per cent of body proteins incorporate progressively greater amounts of test doses of  $\text{S}^{35}$  with increasing protein depletion.

The amino acid composition of hair of African children suffering from

kwashiorkor has been found by Close (179) to be low in total cystine content, but a Central American kwashiorkor case did not show this reduction. The severe retardation in maturation of the bones of the hand in kwashiorkor has been described by Jones & Dean (180), who also find decalcification and many transverse lines indicating past disturbances in growth.

Due largely to work of Brock and his group in South Africa, the doubts as to the primary importance of protein deficiency in producing the basic features of kwashiorkor appear to have been resolved. After the demonstration that "initiation of cure" could be induced not only with skim milk but also with vitamin-free casein plus water, glucose, and salts [Brock *et al.* (181)], studies were begun of the effectiveness of synthetic amino acid combinations for this purpose. Hansen, Howe & Brock (182) first reported that cure could also be initiated by a mixture of 18 synthetic amino acids simulating casein, plus glucose, water and salts with or without vitamin mixtures. Initiation of cure could also be obtained, although less satisfactorily with a mixture of 11 amino acids. It has been made clear that vegetable protein formulas which are as satisfactory as milk for the treatment of kwashiorkor can be developed [Scrimshaw, *et al.* (58); Venkatachalam *et al.* (183); Dean (184) and Thompson (185)]. Close (186) has studied the breast milk of two women in the Belgian Congo whose infants developed kwashiorkor while still nursing and concludes that the milk was insufficient in quantity but not different in its amino acid composition from that of other African mothers.

Balance studies carried out on infants suffering from kwashiorkor at the time of admission and at intervals during treatment are also revealing as to the nature of the syndrome. Senecal *et al.* (187) and Robinson *et al.* (188) have found the intestinal absorption of infants with kwashiorkor receiving different amounts of milk protein to be good and the percentage of retention to remain high for several weeks despite high intakes. Gómez *et al.* (189) also demonstrate strongly positive balances throughout treatment and recovery on *ad libitum* diets of milk, black beans and corn tortillas. In a study of the effect of lysine supplementation of milk, Gómez *et al.* (190) did not call attention to the difference in response between three children with edema (apparently kwashiorkor) and two without edema (apparently marasmus). In the edematous cases nitrogen retention was high and gradually decreased as nitrogen stores were repleted, and no effect of stepwise increases in lysine addition was evident. In the children with no edema, initial retention was low, indicating that relative nitrogen stores were not greatly depleted, but gradually increased as the greatly reduced muscle mass began to fill out. For this reason, no conclusion is justified in those latter cases as to the effect of the gradually increasing lysine supplements.

The need to distinguish between the conditions of kwashiorkor and marasmus has been emphasized by Matsaniotis (165) who found no difference in either the rate of amino acid absorption or intestinal proteolytic activity in 12 "undernourished" children compared with 15 well-nourished children. He considered these results expected since undernourished infants

"although exhibiting a chronic reduction of their protein store, do not suffer from acute protein depletion." The differences between the marasmus and kwashiorkor have also been discussed by Béhar *et al.* (191).

McCarthy (192) believes that the predominance of starchy foods and the correspondingly low protein content of the diets of Polynesians are responsible for the relatively high percentage of liver enlargement in children and cirrhosis in older adults. Fierro del Río, *et al.* (193) showed that in patients with severe malnutrition the infusion of 1 liter of 0.9 per cent NaCl solution caused a marked decrease of total circulating proteins due to a reduction in all fractions as compared with an increase in well-nourished subjects for all except gamma globulin. A paper by Aschkenasy (194) contains a comprehensive review of the blood disorders associated with protein deficiency.

#### STUDIES OF SPECIFIC AMINO ACID DEFICIENCIES

In addition to the many studies of the consequences of protein deficiency discussed in the previous section, a growing number of papers investigating the effects of single amino acid deprivations have appeared. Vohra & Kratzer have shown that lysine deficiency produces hair lighter and finer in texture than normal in rats (195) and feather depigmentation in turkey poults (196). This has been confirmed for chicks of four breeds by Klain *et al.* (197). Apparently, lysine deficiency also results in a decreased skeletal deposition of Ca<sup>45</sup> due at least in part to retarded bone growth [Likins, Bavetta & Posner (198)].

Pair-fed rats given a diet marginally deficient in lysine (0.25, 0.50, and 1.00 per cent) show a lower activity of xanthine oxidase at the 0.25 per cent level. In the group fed the diet with 0.50 per cent of lysine, there was a preferential synthesis of xanthine oxidase over liver protein [Bavetta & Narrod (199)]. Both *ad libitum* and force feeding techniques were used by Van Pilsum, Speyer & Samuels (200) to investigate the effects of diets deficient in tryptophan, isoleucine, or phenylalanine upon the activities of a number of tissue enzymes in rats. The absence of any of these amino acids caused a decrease of liver arginase and aconitase and kidney D-amino acid oxidase parallel to protein loss of the organ, while catalase and xanthine oxidase decreased irrespective of the total protein of the liver.

In the fifth of a series of papers on the histopathology of amino acid deficiency, Scott (201) has studied the effect of the complete lack of isoleucine in rats. The multiple alterations observed in the pituitary, testes, and secondary sex glands were closely similar to those previously observed as a consequence of threonine, histidine, phenylalanine, and tryptophan deficiencies. The changes apparently represent the interference of single amino acid deficiencies with protein metabolism rather than specific effects attributable to the lack of the amino acid itself.

Liver necrosis in rats is easily produced on a diet of either torula or brewer's yeast fed at the 9 per cent level but can be prevented by adding 0.5 per



cent of either DL-methionine or L-cystine [Goyco (202)]. Interestingly enough, one-third brewer's and two-thirds torula yeast, fed together at an 18 per cent protein level, did not have this effect and instead resulted in full liver protein regeneration. Again, it appears that giving enough of a protein of poor biological value will overcome, under certain conditions, the effect of an amino acid deficiency apparent at lower levels of total protein intake.

Hallanger & Schultze (203) show that rats can develop a severe fatty liver during lactation when fed a supposedly complete essential amino acid mixture simulating casein and adequate for growth. This is another indication of the possibility that a ration may be adequate for growth and maintenance but still inadequate to prevent pathological changes in tissues under stress conditions. Harper & Benton (204) found fatty infiltration in rat liver greater on a diet containing 9 per cent casein plus 12 per cent gelatin than on one containing 18 per cent or more casein. This appeared due to the low content of threonine and the high content of arginine and glycine in gelatin. Arata *et al.* (205) concluded that both a defect in diphosphopyridine nucleotide production and improper metabolism of endogenous diphosphopyridine nucleotide in liver are major factors in the accumulation of liver fat which results from partial threonine deficiency in the rat.



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## ENZYMATIC METABOLISM OF DRUGS AND OTHER FOREIGN COMPOUNDS<sup>1,2,3</sup>

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In recent years it has become evident that urinary excretion is of relatively minor importance in limiting the duration of action of drugs; and that most therapeutic agents cannot be excreted except in minor amounts until they undergo chemical modification. Without biochemical mechanisms for the metabolism of foreign compounds, much of our present drug therapy would be dangerous and impracticable, since the action of most therapeutic agents would persist for too long a time. In addition, treatments for poisoning by many toxic organic compounds would be almost hopeless. Mechanisms of drug inactivation are therefore of major importance.

The many studies on the fate of foreign compounds in the body have shown that the metabolism of a multitude of drugs and related substances follows surprisingly few chemical pathways. Recently, important advances have been made towards an understanding of the enzymatic processes that carry out a number of these metabolic alterations; this has been made possible by the development of simple, general procedures for the estimation of organic compounds in microgram amounts.

This review is concerned mainly with the characterization of enzymes responsible for drug metabolism, their possible mode of action, and their normal function in the body. Many important contributions on the fate of individual drugs *in vivo* must be passed over. What would otherwise have been a serious omission will be well taken care of by the publication of the second volume of *Detoxication Mechanisms* by R. T. Williams, scheduled to appear about the same time as the present article.

### OXIDATION BY MICROSOMAL ENZYME SYSTEMS

Considerable progress has been made recently in an understanding of

<sup>1</sup> The survey of the literature pertaining to this review was completed in November, 1957.

<sup>2</sup> The following abbreviations are used in this chapter: ATP for adenosine triphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; FAD for flavin-adenine-dinucleotide; FMN for flavin mononucleotide; TPNH for reduced triphosphopyridine nucleotide; UDP for uridine diphosphate; and UDPGA for uridine diphosphate glucuronic acid.

<sup>3</sup> The term "foreign compound" is used in this article to specify those organic compounds found in plants or made synthetically which are not normally present in the body.

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The many studies on the fate of foreign compounds in the body have shown that the metabolism of a multitude of drugs and related substances follows surprisingly few chemical pathways. Recently, important advances have been made towards an understanding of the enzymatic processes that carry out a number of these metabolic alterations; this has been made possible by the development of simple, general procedures for the estimation of organic compounds in microgram amounts.

This review is concerned mainly with the characterization of enzymes responsible for drug metabolism, their possible mode of action, and their normal function in the body. Many important contributions on the fate of individual drugs *in vivo* must be passed over. What would otherwise have been a serious omission will be well taken care of by the publication of the second volume of *Detoxication Mechanisms* by R. T. Williams, scheduled to appear about the same time as the present article.

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the enzyme systems that oxidize drugs and other foreign compounds. A large number of foreign compounds are oxidized along a variety of metabolic pathways by enzyme systems in liver microsomes. In view of their unusual requirements for both TPNH and oxygen, the microsomal systems will be discussed as a group.

**Deamination.**—The discovery of a microsomal enzyme system that deaminates amines stems from the observation of Axelrod (1) that rats and dogs convert amphetamine to *p*-hydroxyamphetamine, but rabbit liver microsomes deaminate amphetamine to yield phenylacetone and ammonia (2). Historically this was the first of a series of oxidative microsomal systems shown to require both oxygen and TPNH.

The deaminase activity of rabbit microsomes is markedly depressed when rat microsomes are added, suggesting that the low activity in dog and rat microsomes is due to inhibitory factors rather than to a deficiency of enzymes. However, the inhibition may result from a decrease in TPNH due to the action of TPNH oxidase present in the added microsomes [Gillette *et al.* (3)]. Axelrod also presented evidence for a heat stable activator in microsomes, but the nature of this factor is not known.

The microsomal deaminase differs from monoamine oxidase, not only in its localization and cofactor requirement, but in its substrate specificity. Thus, the microsomal enzyme deaminates methylamphetamine, amphetamine, and ephedrine, compounds that inhibit monoamine oxidase, but does not act on monoamine oxidase substrates like tyramine, benzylamine and isoamylamine. The *L*-isomers of amphetamine and ephedrine are metabolized considerably faster than the *D*-isomers (2). It may be of importance that the substrates for the microsomal enzyme are lipid soluble.

The presence in rabbit liver of two deaminating systems with different substrate specificities could lead to confusion in studies of monoamine oxidase. For example, older studies have indicated that monoamine oxidase in rabbits, but not in other species, deaminates mescaline [Blaschko (4)]. This observation can be explained if mescaline like amphetamine is metabolized by rabbit liver microsomes.

**N-Dealkylation.**—The oxidative removal of N-alkyl groups from drugs is a rather general reaction and considerable advances have been made in outlining the enzyme systems involved. Mueller & Miller (5) observed that 4-dimethylaminoazobenzene and related dyes are demethylated by a rat liver enzyme system which requires TPN, DPN, and oxygen. The reaction does not involve transmethylation since it yields formaldehyde. Recently, La Du *et al.* (6) showed that N-dealkylation is accomplished in rabbit liver microsomes by a mechanism that is TPNH dependent and requires oxygen. The system removes methyl, ethyl, and butyl groups; accordingly, monomethyl-4-aminoantipyrine and its ethyl and butyl analogues are converted to 4-aminoantipyrine, the methyl group appearing as formaldehyde and the ethyl group as acetaldehyde. The microsomal mechanism dealkylates a variety of N-alkylamines, including methylamphetamine, meperidine (Demerol),

methylaniline, ethylaniline, quinacrine (Atabrine) (6), diacetylmorphine (heroin), methadone, and codeine [Axelrod (7)].

Despite the fact that the microsomal demethylation system acts on many foreign substances, it does not promote the dealkylation of normally occurring alkylamines like sarcosine or dimethylaminoethanol. Conversely, the mitochondrial enzyme responsible for the demethylation of sarcosine does not act on foreign alkylamines [Gaudette & Brodie (8)].

An unusual type of dealkylation is suggested from the work of McKennis *et al.* (9), who isolated pyridyl- $\gamma$ -N-methylaminobutyric acid from the urine of dogs given C<sup>14</sup> labelled nicotine. Hucker (10) demonstrated that nicotine is metabolized in liver microsomes by a TPNH dependent enzyme system. No evidence for the formation of nornicotine was found. This suggests that the alkaloid is first oxidized in the pyrrolidine ring to form the 2-hydroxy derivative which is at the same stage of oxidation as the proposed hydroxymethyl intermediates in the dealkylation of alkylamines (*vide infra*). A similar type of mechanism may also be involved in the splitting of other saturated ring systems containing nitrogen.

The enzyme system that removes methyl groups may differ from that which removes higher alkyl groups.  $\beta$ -Diethylaminoethyl diphenylpropylacetate (SKF 525-A) antagonizes the demethylation of monomethyl-4-aminoantipyrine, meperidine and several other methylated amines, but does not interfere with the dealkylation of the ethyl and butyl homologues of 4-aminoantipyrine. Furthermore, the relative rates of microsomal dealkylation for monomethyl-4-aminoantipyrine and meperidine are the same among a number of species, but the relative rates for monomethyl-4-aminoantipyrine and its ethyl analogue are different (8). Evidence for the presence of more than one demethylating enzyme in microsomes has also been cited (7, 8).

The mechanism of oxidative dealkylation has been the subject of a number of studies. Horning and his associates postulate that N-oxides may be intermediate products [Fish *et al.* (11, 12)]. They have shown that liver homogenates supplemented with DPN, AMP, and nicotinamide catalyze the rearrangement of N,N-dimethyl tyrosine oxide and N,N-dimethyltryptophan oxide to yield formaldehyde and the secondary amines. This system also catalyzes the oxidation of N,N-dimethyltryptamine to the corresponding N-oxide. From these observations the authors have proposed that the dealkylation of alkylamines may proceed along the following pathway:



This interesting theory merits further attention. In this connection Gillette<sup>4</sup> has shown that the N-oxide of dimethylaniline is dealkylated by liver microsomes much more slowly than is dimethylaniline. This by no means eliminates the nitrogen oxide theory from consideration since the highly polar nitrogen oxide may have difficulty in penetrating into microsomes.

<sup>4</sup> Unpublished observation.

The recent work of O'Brien (13) provides an example of N-oxide formation in mouse microsomes. The insecticide Schradan (octamethylpyrophosphoramide), which is employed as an insecticide for plants and was a drug for the treatment of myasthenia gravis, is oxidized by liver microsomes to a powerful inhibitor of cholinesterase. DPNH and catalase are required in the reaction [see also Davison (119)].

*O-Dealkylation.*—Aromatic ethers such as *p*-ethoxyacetanilide (phenacetin) and codeine undergo O-dealkylation in the body to form phenols (14, 15).

Bray *et al.* (16) demonstrated that the demethylation of substituted anisoles occurs in rabbit liver slices and that the methyl group is oxidatively split to yield formaldehyde. Recently Axelrod (17) showed that the ether cleavage enzyme system is localized in rabbit liver microsomes and that it requires TPNH and oxygen. The products of the reaction are a phenol and an aldehyde; codeine is oxidized to morphine and formaldehyde, and *p*-ethoxyacetanilide is converted to *p*-hydroxyacetanilide and acetaldehyde. Among other substrates dealkylated by this enzyme system are papaverine, quinine, colchicine, and mescaline.

Axelrod suggested that more than one ether cleavage enzyme system is present in liver microsomes. Rabbit and guinea pig microsomes metabolize *p*-ethoxyacetanilide equally well, but rabbit microsomes convert codeine into morphine about ten times faster than guinea pig microsomes. Furthermore,  $\beta$ -diethylaminoethyl diphenylpropylacetate (SKF 525-A) markedly inhibits the cleavage of codeine but not of *p*-ethoxyacetanilide.

*Oxidation of thioethers.*—Thioethers are not usually dealkylated *in vivo*, but are oxidized to the corresponding sulfoxide derivatives. For example, phenothiazine is converted to a sulfoxide by calves and sheep [Clare (18)]. Burns *et al.* (19) have shown that 4(phenyl-thioethyl)-1,2-diphenyl-3,5-pyrazolidinedione (G-25671), an antirheumatic agent, is oxidized in man to a sulfoxide. This derivative accounts for the uricosuric action of the parent compound (G-25671) and is in fact the most potent inhibitor of tubular reabsorption of uric acid hitherto reported. Chlorpromazine, a phenothiazine derivative, is also metabolized to a sulfoxide in dog and man (20).

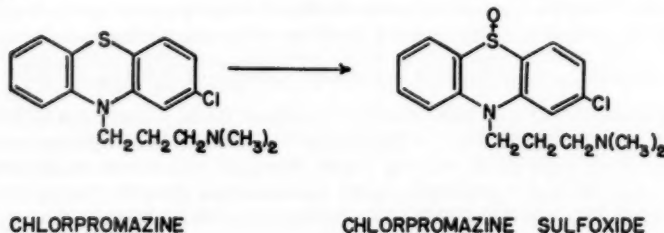


FIG. 1.

Kamm *et al.* (21) have shown that chlorpromazine is oxidized to its sulfoxide derivative by a TPNH dependent enzyme system located in guinea pig liver microsomes. It is not yet known whether chlorpromazine sulfoxide is further oxidized to the sulfone, but Rose & Spinks (22) have shown that methylthioaniline is oxidized in mice to the corresponding sulfone.

Under anaerobic conditions, chlorpromazine sulfoxide but not the sulfone is reduced to chlorpromazine by guinea pig liver homogenates (21), in conformity with the prediction of Williams (23) that sulfoxides can be reduced in the body, but not sulfones.

*Hydroxylation of aromatic rings.*—Considerable light has been thrown on the biochemical mechanism responsible for the hydroxylation of aromatic drugs. Mitoma *et al.* (24) showed that acetanilide is hydroxylated in rabbit liver by a microsomal enzyme system that requires oxygen and TPNH. A number of other aromatic compounds are hydroxylated by liver microsomes including aniline, salicylic acid, naphthalene, quinoline, diphenyl, 2-naphthylamine, 2-aminofluorene, benzene, and 3,4-benzpyrene (24, 25, 26). A number of normally occurring substrates, including L-phenylalanine, L-tryptophan, kynurenine, and tryptamine form phenolic derivatives in the body but none of these is hydroxylated by the microsomal enzyme system (24).

Mitoma *et al.* (24) also reported that microsomes hydroxylate aromatic ring systems in more than one position. Thus, acetanilide yields mainly the *p*-hydroxy derivative but also traces of the *o*- and *m*-isomers. Quinoline yields 3-hydroxyquinoline as the major product with traces of the 6- and 7-hydroxyquinolines. Parke & Williams (27) suggested that ortho and para hydroxylation may involve different enzymes. Thus, rabbits given aniline excrete six times as much *p*-hydroxyaniline as the ortho isomer. In contrast, cats excrete two to three times as much of the ortho as the para isomer. Posner (28) demonstrated a similar type of species difference in the hydroxylation of acetanilide. Only slight amounts of *o*-hydroxyacetanilide are formed by rabbit liver microsomes, but considerable amounts are formed by those of cat liver. If different enzyme systems are required for directing hydroxylation in the different positions of an aromatic ring, then it may not be possible to formulate rigid rules of substitution; furthermore, rules for describing the orientation of hydroxylation in one species may not apply to another or even to another strain of the same species.

There is evidence that a dihydro-diol compound may be an intermediate in the formation of phenols *in vivo*. For example, rats and other mammals receiving naphthalene excrete 1,2-dihydronaphthalene 1,2-diol (29), suggesting that this compound may be an intermediate in the formation of 1-naphthol. Recently, Booth & Boyland (25) demonstrated that rat liver microsomes oxidize naphthalene to yield both 1,2-dihydronaphthalene-1,2-diol and 1-naphthol; and Posner *et al.* (30) have shown that rabbit microsomes convert quinoline to a dihydroquinoline-diol and 3-hydroxyquinoline by oxidative reactions requiring TPNH. It is possible, however, that the dihydro-diol is



not the precursor of 1-naphthol since incubation of the dihydro-diol with microsomes does not convert it to 1-naphthol (25).

Indirect evidence indicates that certain catechols may be formed from dihydro-diol intermediates. For example, a catechol is excreted in only trace amounts after dosing rabbits with *p*-chlorophenol whereas 4-chlorocatechol is a major metabolite after giving chlorobenzene. Smith *et al.* (31) suggest that the catechol is formed according to the following reactions:

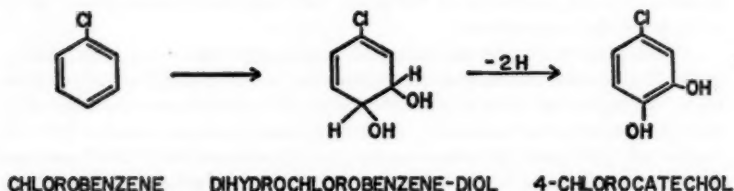


FIG. 2

They isolated from urine the postulated dihydro-diol intermediate, 3,4-dihydro-3,4-dihydroxychlorobenzene. However, if dihydro-diols are precursors of catechols the presence of a tissue dihydro-diol dehydrogenase should be demonstrable.

A number of years ago Knox (32) purified a rabbit liver enzyme system which hydroxylates quinolines in the 2 position. This system is obviously different from that in the microsomes since it specifically acts on heterocyclic compounds with an active  $\alpha$ -hydrogen, is a flavoprotein that can react with quinoline anaerobically, and is intimately associated with liver aldehyde oxidase. The reaction may be formulated as follows:

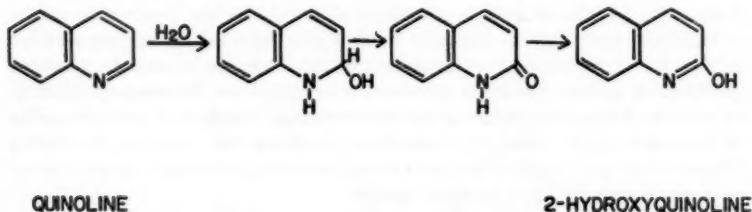


FIG. 3

If quinoline is considered as a cyclic aldehyde in the form of a Schiff base, this type of reaction could be considered as oxidation of the cyclic aldehyde to form a lactam rather than hydroxylation in the usual sense. A similar mechanism may explain the dual function of xanthine oxidase in oxidizing purines and aldehydes and has been postulated by Bergmann & Dikstein (33).

*Oxidation of alkyl hydrocarbon chains.*—A number of important drugs, barbiturates for example, are inactivated mainly by sidechain oxidation. Oxidation at both terminal ( $\omega$ ) and penultimate ( $\omega-1$ ) carbon atoms has been demonstrated with the barbiturates, thiopental (34) and pentobarbital (35), while oxidation in the  $\alpha$  position is less common and appears to be restricted to compounds such as ethyl, propyl, and butylbenzene (36), which have an activated carbon atom next to an aromatic ring.

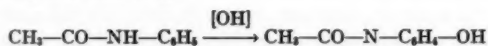
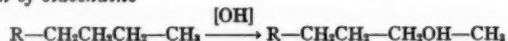
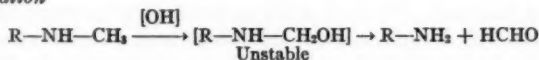
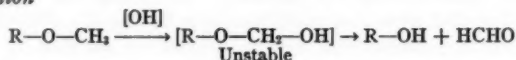
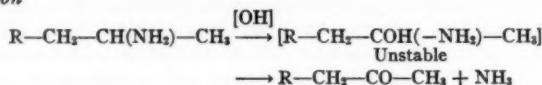
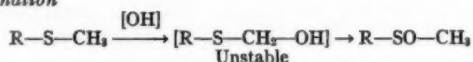
Gould & Shideman (37) studied the metabolism of barbiturates in tissue homogenates. The disappearance of thiopental from a rat liver homogenate required the addition of nicotinamide, ATP, cytochrome-*c* and a Krebs cycle intermediate. More definitive information concerning the metabolism of barbiturates resulted from studies of Cooper & Brodie (38, 39), who showed that hexobarbital, pentobarbital, and thiopental are oxidized by the action of enzymes in microsomes that have the requirement for both TPNH and oxygen. Products of the reactions are isolated from rabbit liver homogenates: hexobarbital yields a keto-hexobarbital; pentobarbital forms about equal amounts of an alcohol (penultimate oxidation) and an acid (terminal oxidation); thiopental yields mainly a compound with a carboxylic acid on the terminal carbon together with a small amount of an alcohol (penultimate oxidation). Recently Gillette (40) demonstrated that rabbit liver microsomes oxidize thiopental at the terminal carbon atom to yield a primary alcohol and that further oxidation to the acid is accomplished by enzymes in the soluble part of the cell.

The number of enzyme systems needed to hydroxylate alkyl sidechains is not known. However, different systems are involved in the oxidation of oxybarbiturates and thiobarbiturates for the former compounds are oxidized only by liver while thiobarbiturates are also metabolized by kidney, brain, and heart. Rabbit kidney also oxidizes thiopental in the terminal carbon atom with an enzyme system requiring TPNH and oxygen (39). It would not be surprising if there are different enzymes to oxidize terminal and penultimate carbon atoms of the same compound.

*General.*—The preceding sections have described the oxidation of a large number of foreign compounds by a diversity of enzyme systems located in liver microsomes. Common requirements for TPNH and oxygen suggest that they are closely related. The apparent paradox of systems requiring TPNH in an oxidative reaction is difficult to explain; and a detailed study of the mechanism has been hampered by the difficulty of obtaining the enzymes in a soluble form.

In recent years a number of other enzyme systems have been shown to require a reduced pyridine nucleotide and oxygen. These requirements are generally associated with biochemical mechanisms that hydroxylate aromatic or aliphatic ring systems [Mason (41)].

The various oxidative pathways in microsomes may all be written as hydroxylation reactions, that is, the direct substitution of a hydroxyl group for a hydrogen.

*Hydroxylation of aromatic rings**Oxidation of sidechains**N-Dealkylation**O-Dealkylation**Deamination**Sulfoxide formation*

The first step in the oxidation of the last four reactions may also be written as a direct oxidation to the oxides of N, O and S, as proposed by Fish *et al.* (11, 12) for the dealkylation of N-alkylamines.

A common step in microsomal oxidation may be the formation of an intermediate "hydroxyl" donor, which in conjunction with a number of non-specific enzymes can transfer a hydroxyl radical to an appropriate acceptor substrate.

A hydrogen peroxide-ferrous iron reagent is known to produce free radicals capable of slowly hydroxylating aromatic compounds (42). Udenfriend *et al.* (43) found a greatly increased rate of hydroxylation, with a model system consisting of ascorbic acid, oxygen, and inorganic iron chelated with Versene. At least two of the microsomal oxidative reactions, aromatic hydroxylation and O-dealkylation, are achieved by the same model system [Brodie *et al.* (44)]. Barbiturates, alkylamines, and free amines are also oxidized by the system, but the expected end products are unstable in the reaction mixture and no conclusion can be drawn as to whether sidechain oxidation, dealkylation and deamination are also effected by the model system. Udenfriend *et al.* (43) found evidence for the formation of a  $\text{H}_2\text{O}_2$ -ascorbic acid complex and suggested that this complex together with inorganic iron generates active hydroxyl groups which exchange with ring hydrogen to form phenols. A somewhat different concept has been proposed by Mason & Onoprienko (45).

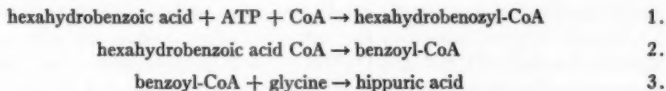
Peroxidase model systems consisting of enzymatically produced  $H_2O_2$  and hemoporphyrin proteins like methemoglobin, cytochrome-c, or horse-radish peroxidase dealkylate monomethyl-4-aminoantipyrine and N-methylaniline to the free amines and formaldehyde [Gillette *et al.* (46)]. Evidence was found of an intermediate substance presumably the hydroxyl methyl derivative. Similarly, horse-radish peroxidase or catalase together with  $H_2O_2$  converts chlorpromazine to its sulfoxide [Cavanaugh (47)].

The requirement for TPNH and oxygen in the microsomal oxidation of foreign compounds suggests that a peroxide may be formed in these reactions as well. In fact, Gillette *et al.* (3) have reported that TPNH is oxidized by liver microsomes even in the absence of drug substrate to produce "peroxide." The oxidation of TPNH is presumably an integral part of the reaction, since inhibitors of TPNH oxidation equally inhibit the dealkylation of monomethyl-4-aminoantipyrine. The authors have considered the possibility that the peroxide together with a variety of peroxidases might be responsible for the various types of oxidative reactions. Evidence against the view that the drug-oxidative systems of liver microsomes utilize free hydrogen peroxide is the failure of cyanide to affect appreciably the oxidation of hexobarbital or the demethylation of monomethyl-4-aminoantipyrine. Furthermore, a hydrogen peroxide generating system cannot replace the requirement for TPNH in the various oxidative pathways. The authors have speculated whether an organic peroxide might be the intermediate which reacts with enzyme and drug substrate, even though hydrogen peroxide does not.

#### OTHER TYPES OF OXIDATION

*Aromatization of hydroaromatic compounds.*—A number of foreign cyclohexane ring acids are converted *in vivo* to aromatic acids (48). For example, hexahydrobenzoic acid is excreted by several species as benzoic or hippuric acid. Beer *et al.* (49) showed that hexahydrobenzoic acid and the three possible tetrahydrobenzoic acids are aromatized by rabbit liver slices but not by tissue homogenates.

Mitoma *et al.* (50) have shown that aromatization of hexahydrobenzoic acid occurs in liver mitochondria of the guinea pig in the presence of oxygen,  $\alpha$ -ketoglutarate,  $Mg^{++}$ , glycine, ATP and cytochrome-c. They describe the following sequence of reactions:



Reactions 1 and 2 are reminiscent of the activation and oxidation of fatty acids. Reaction 3 transfers the benzoyl group to glycine thereby removing benzoic acid which inhibits step 1. There is a marked species difference in the activity of the aromatization system. Thus guinea pig and rabbit liver mitochondria are much more active than rat mitochondria, while no activity is detectable in mitochondria of cat, mouse, dog, monkey, and man.

*Oxidation of alcohols and aldehydes.*—Recent work indicates the importance of alcohol and aldehyde dehydrogenases in the metabolism of a number of drugs that are converted to an alcohol as an intermediate. Gillette (40) has shown that rabbit liver microsomes oxidize *p*-nitrotoluene to *p*-nitrobenzyl alcohol by a TPNH dependent enzyme system, and that further oxidation to *p*-nitrobenzoic acid requires DPN-dependent alcohol and aldehyde dehydrogenases in the soluble fraction of liver. Reference has been made in the section on the oxidation of alkyl sidechains to the formation of the intermediate alcohol in the oxidation of thiopental to thiopental carboxylic acid.

Aldehydes can be oxidized to the corresponding carboxylic acid by at least three mammalian enzymes; a DPN-dependent aldehyde dehydrogenase, xanthine oxidase, and aldehyde oxidase. Aldehyde dehydrogenase acts *in vitro* on many foreign aldehydes (51); and the same enzyme may catalyze the oxidation of the aldehydes formed by the action of monoamine oxidase on 5-hydroxytryptamine, histamine, epinephrine and norepinephrine (52). Xanthine oxidase and aldehyde oxidase also attack a variety of foreign aldehydes but their relative importance in the metabolism of foreign compounds remains to be clarified.

Cooper (53) has reported that the oxidation of chloral hydrate to trichloroacetic acid by rabbit, rat and beef liver is catalyzed by a DPN-dependent enzyme that is not the usual aldehyde dehydrogenase. The purified system acted on chloral hydrate but was inactive toward a considerable number of other aldehydes.

#### REDUCTION

*Nitro reductase.*—Chloramphenicol is one of the few drugs that are aromatic nitro compounds, but a number of nitro compounds are used industrially as chemical intermediates, many of which are slowly reduced in the body to the corresponding amine.

Fouts & Brodie (54) have shown that the system that reduces chloramphenicol, *p*-nitrobenzoic acid, etc. is present in a number of rabbit tissues, of which liver is the most active. The nitro reductase is present both in the soluble fraction of the cells and in microsomes. Though the enzyme is highly active under anaerobic conditions, it is much less effective aerobically, which probably explains why the reduction of nitro compounds is usually so slow *in vivo*. Both TPNH and DPNH act as hydrogen donors, but the former is considerably more effective. The system is presumably a flavoprotein, since activity is lost on mild acid treatment and restored by FAD. But its properties differ from other known flavoproteins since FMN and riboflavin can also restore the activity. Furthermore, any of the three flavins added in excess to the liver homogenate markedly accelerates the normal activity of nitro reductase. The authors have postulated that the excess flavins may

act as an artificial electron carrier between the reduced pyridine nucleotide and substrate.

There is considerable species difference in the activity of the reductase; the activity of mice, guinea pigs and rabbits is much greater than that of rats and dogs.

Kielley (55) has reported that xanthine oxidase slowly catalyzes the reduction of nitrophenols. On the other hand, Fouts & Brodie (54) found that 2,4-dinitrophenol and *p*-nitrophenol are not metabolized by nitro reductase but instead inhibit the enzyme; and that xanthine oxidase does not act on *p*-nitrobenzoic acid. It may be concluded that the reduction of nitrophenols and of nonphenolic nitro compounds are effected by different mechanisms. As further evidence, Westerfeld *et al.* (56) have shown that the activity of chick liver homogenates in reducing 2,4-dinitrophenol is more stable to heat than the activity in reducing *p*-nitrobenzene sulfonamide. They report that treatment of a chicken liver xanthine oxidase preparation with Pangestin destroys the ability to reduce phenols without affecting xanthine oxidase activity.

Aromatic nitro compounds can also be reduced by bacteria present in the large intestine (57), which must be considered when studying the fate of orally administered nitro compounds. The nitro reductase of *Escherichia coli* differs in a number of respects from mammalian nitro reductase; it is not inhibited by air, it requires cysteine and  $Mn^{++}$ , and it is inhibited by chlor-tetracycline (Aureomycin) and other chelating agents (58, 59, 60).

The cardiovascular drugs, nitroglycerin and erythritol tetranitrate, which are aliphatic nitrate esters, are decomposed in various tissues to yield inorganic nitrite. Heppel & Hilmoe (61) have purified an enzyme from hog liver acetone that catalyzes the interaction of these nitrates with glutathione to form inorganic nitrite. They postulate that the reduction of the nitrate esters precedes hydrolysis.

*Azo reductase.*—Azo compounds are reductively split in the body to form primary amines. The reaction presumably occurs in two steps; the formation of a hydrazo compound, followed by reductive splitting of the N—N bond [Bray *et al.* (62)].

In 1950 Mueller & Miller (63) showed that 4-dimethylaminoazobenzene is reductively split by a TPNH-dependent enzyme system present mainly in rat liver microsomes. The enzyme system is a flavoprotein having FAD as a prosthetic group.

Recently, Fouts *et al.* (64) studied the reduction in rabbit tissues of a number of azo compounds including prontosil and 4-dimethylaminoazobenzene. Azo reductase activity is present in a number of tissues of which liver is the most active. The enzyme is like nitro reductase in a number of ways; it is localized mainly in the soluble fraction of rabbit liver, it has the same relative activity among various rabbit tissues and among the livers of different species, and its activity is enhanced by the addition of large amounts

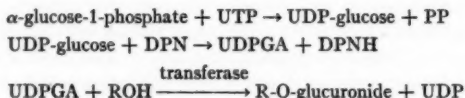
of riboflavin, FMN or FAD. However, the fact that azo reductase can be separated from nitro reductase by ammonium sulfate fractionation indicates that they are different enzymes. Azo reductase also differs from nitro reductase in that it is specifically dependent on TPNH and it retains most of its activity under aerobic conditions.

### CONJUGATION

The term "conjugation" includes the synthesis of ethereal sulfates, glucuronides, mercapturic acids, amino acid conjugates, and acetylated amines.

*Glucuronide conjugation.*—Phenols, alcohols and carboxylic acids form glucuronides, the hydroxyl compounds forming ethers and the acids yielding esters (65). Recently, the formation of N- and S-glucuronides has also been reported [Boyland *et al.* (66); Clapp (67)].

The mechanism of glucuronide formation has now been virtually established. Glucuronic acid does not conjugate directly but must be present in an active form, uridine diphosphate glucuronic acid (UDPGA). Glucuronic acid is transferred from UDPGA to an acceptor compound by a transferase enzyme. The sequence of events, starting from glucose-1-phosphate, is as follows:



The product is a  $\beta$ -glucuronide and therefore must involve an inversion of the  $\alpha$  linkage of glucuronic acid in UDPGA.

UDPGA is synthesized in the soluble fraction of the liver (68); and the transferase enzyme is present in the microsomes [Dutton (69)]. This latter enzyme, which has been solubilized and purified several fold by Isselbacher (70), has no  $\beta$ -glucuronidase activity. Axelrod *et al.* (71) find that the same microsomal transferase forms the N-glucuronides of aniline, *p*-phenetidine and *p*-toluidine.

Drugs can stimulate an increased urinary excretion of glucuronic acid that is unrelated to their biotransformation. Burns *et al.* (72) have reported that many drugs, including barbital which is not metabolized *in vivo*, induce a pronounced increase in the excretion of free D-glucuronic acid. The synthesis of D-glucuronic acid under these conditions appears to involve a mechanism other than the transfer of glucuronic acid from UDPGA.

The function of  $\beta$ -glucuronidase remains obscure. It hydrolyzes  $\beta$ -glucuronides but what purpose this serves is not clear. In addition it can catalyze the transfer of glucuronic acid from one glucuronide to another [Fishman & Green (73)]. This reaction may have an important physiologic role but its importance in drug metabolism remains to be established.

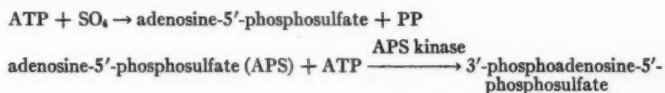
*Sulfate conjugation.*—An important pathway in the metabolism of foreign



phenols is the formation of sulfate esters. Conjugation with sulfate is not limited to phenols, as aromatic amines can also form sulfamates (66).

The synthesis of an ethereal sulfate from a phenol and inorganic sulfate has been achieved in rat liver homogenates. The complete system is present in the soluble fraction and the synthesis takes place in two steps. The first requires ATP and transforms inorganic sulfate to "active sulfate"; the second transfers sulfate to a phenol [see Fishman (74)].

Robbins & Lipmann (75) isolated "active sulfate" from liver extracts and identified it as 3'-phosphoadenosine-5'-phosphosulfate. They showed that it is formed by yeast extracts as follows:

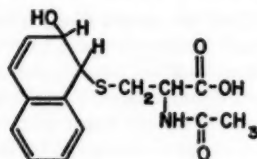


Little is known about the enzyme that transports sulfate from 3'-phosphoadenosine-5'-phosphosulfate to its acceptor. Segal (76) has shown that about four phosphate ions are formed for each sulfate ester in the over-all reaction, but the significance of this is not clear. Gregory & Nose (77) have demonstrated that there are at least two transferases in liver, one for the transfer of sulfate from 3'-phosphoadenosine-5'-phosphosulfate to  $\beta$ -hydroxy steroids and the other for the transfer of sulfate to phenols and estrogens. A transferase in chicken embryos [D'Abramo & Lipmann (78)] catalyzes the transfer of sulfate from 3'-phosphoadenosine-5'-phosphosulfate to chondroitin and requires either ATP or UTP for maximum activity.

*Mercapturic acid conjugation.*—Aromatic compounds are converted to mercapturic acids in a number of ways. The acetylcysteinyl group can replace a nuclear hydrogen, as in naphthalene (79); an active chlorine as in 2:4-dichloronitrobenzene or benzyl chloride (80, 81); a labile nitro group as in pentachloronitrobenzene (82).

Aromatic compounds react with cysteinyl groups bound in peptides rather than directly with free cysteine [Gutmann & Wood (83)]. The conjugate is thought to be hydrolyzed to yield the arylcysteine which is then acetylated to form the mercapturic acids [Gutmann & Wood (84)]. This view is strengthened by the observation of Bray & Franklin (85) that liver homogenates hydrolyze S-(*p*-chlorophenyl)-glutathione to S-(*p*-chlorophenyl) cysteine. Furthermore, Mills & Wood (86) have isolated *p*-iodophenylcysteine from liver slices incubated with iodobenzene.

Recently, Boyland & Sims (87) isolated a new type of metabolite from the urine of dogs given naphthalene, probably N-acetyl-S-(2-hydroxy-1,2-dihydronaphthyl)-L-cysteine. In acid solution it is immediately decomposed to 1-naphthylmercapturic acid. Since no naphthylmercapturic acid could be detected in the unacidified urines, the authors have suggested that the naphthalene mercapturic acid previously reported as a urinary excretion product of naphthalene is an artifact.



N-ACETYL-S (2-HYDROXY-1,2-DIHYDRONAPHTHYL)-L-CYSTEINE

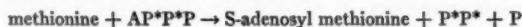
FIG. 4

Knight & Young (88) have found similar acid-labile "premercapturic acids" in the urine of animals fed benzene, naphthalene, anthracene, fluorobenzene, chlorobenzene, bromobenzene and iodobenzene. They noted, however, that benzyl chloride is excreted directly as the mercapturic acid.

**Acylation.**—Acylation involves the conjugation of carboxylic acids with amines. In these reactions an acid is activated by forming a CoA derivative and is then transferred to an acceptor amine. Either the acid or the acceptor amine may be the foreign compound. Thus acetyl-CoA reacts with sulfanilamide and other foreign amines to form acetylated products (89). Benzoic acid also forms an active CoA derivative; the benzoyl radical is transferred to glycine by an enzyme present in kidney and liver mitochondria (90). Glycine is the only amino acid that serves as an acceptor, but the enzyme shows a broad substrate specificity with respect to acyl-CoA donors. Moldave & Meister (91) recently studied the conjugation of phenylacetyl CoA with glutamine and showed that the transferase enzyme is present in human liver and kidney, but is absent from rat and beef tissues. However, rat or beef liver mitochondria supplemented with the purified transferase enzyme from human kidney catalyzes the formation of phenylacetylglutamine (92).

Moldave & Meister (92) showed that adenylyl phenylacetate and adenylyl benzoate are intermediates in the formation of benzoyl-CoA and phenylacetyl-CoA. Thus it is likely that the CoA derivatives of these compounds are formed by the same type of mechanism that forms the CoA derivatives of acetic acid [Berg (93)] and of fatty acids [Jencks & Lipmann (94)].

**Methylation.**—N-Methylation is not an important reaction in drug metabolism but occurs to a minor extent with compounds like pyridine and quinoline. The reaction is not the reverse of demethylation, but is possibly the same as that which catalyzes the transfer of the methyl group of methionine to nicotinamide in forming N-methyl nicotinamide. Cantoni (95, 96) has shown that the latter compound forms in liver and that the reaction requires the presence of "active methionine," identified as S-adenosyl methionine (96) and formed as follows (97):



Armstrong *et al.* (98) observed that 3-methoxy-4-hydroxy mandelic acid is the major metabolite of norepinephrine and epinephrine. Axelrod (99) has shown that a transferase in mammalian liver can conjugate the methyl group of S-adenosyl methionine with the 3-hydroxy group of 3,4-dihydroxy benzoic acid or with the 3-hydroxy group of epinephrine and norepinephrine. Whether methylation of the catechol amines released *in vivo* occurs before or after their deamination is still unsettled.

### HYDROLYSIS

A number of enzymes split their substrates by the addition of water. The present discussion is limited to the hydrolysis of esters and amides, many of which are used as drugs.

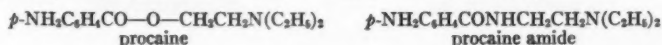
*De-esterification.*—Plasma contains esterases that hydrolyze a number of drugs, including procaine and succinylcholine. Procaine is de-esterified so rapidly in human plasma that the administered drug is metabolized mainly in the blood stream; but it is hydrolyzed much more slowly in plasma of other species [Aven *et al.* (100)]. Procaine (101) and succinylcholine (102) are said to be hydrolyzed by nonspecific plasma cholinesterase which may be a mixture of enzymes. Other drugs, for example meperidine (Demerol) are split in liver but not in plasma [Way *et al.* (103)].

Certain species differences make it likely that a considerable number of esterases may be involved in hydrolyzing foreign esters. For example, rabbit, but not dog or man, has an esterase that de-esterifies ethyl bis(4-hydroxycoumarinyl) acetate (Tromexan) (104). Atropine is stable in human plasma, but is rapidly decomposed by an esterase in rabbit plasma [Godeaux & Tonnesen (105)]. Furthermore, both cocaine and  $\alpha$ -cocaine are hydrolyzed by rabbit plasma whereas the plasma of man and horse can hydrolyze only  $\alpha$ -cocaine [Blaschko *et al.* (106)].

Since succinic acid esters are rapidly hydrolyzed by human plasma, the sodium salts of succinic acid esters can be used to solubilize insoluble alcohols which exert a therapeutic effect. These esters may be given parenterally and they are rapidly hydrolyzed to the active alcohols. For example, chloramphenicol may be given intravenously as the sodium succinyl ester [Glazko *et al.* (107)].

*Deamidation.*—Bray *et al.* (108) reported that a number of amides are hydrolyzed by rabbit liver homogenates, but the reaction is slow compared with the hydrolysis of esters. For example, procaine amide (109) and salicylamide (110) are only slowly hydrolyzed in the body.

Cognizance of the stability of amides has led to the development of useful drugs. For example, procaine, a local anesthetic, inhibits heart arrhythmias but is too rapidly hydrolyzed *in vivo* to have more than a restricted value for this purpose. However, procaine amide which is also a powerful antiarrhythmic agent, is far more stable in the body (109).



## DEHALOGENATION

A number of important halogenated compounds used as insecticides and industrial solvents can lose halogen in the body in various ways, including replacement of the chlorine atom by a hydroxyl group, the loss of hydrogen halide and replacement of chlorine by an acetyl-cysteine group. The latter reaction has been taken up under mercapturic acids.

A number of years ago Heppel & Porterfield (111) reported that dichloromethane, dibromomethane and chlorobromomethane are dehalogenated enzymatically by rat liver yielding formaldehyde and halide ions. Dialyzed extracts or ammonium sulfate fractions of liver require cyanide and either glutathione or cysteine for activation and the reaction is inhibited by sodium fluoride. Bray *et al.* (112) demonstrated that boiled tissue extracts react with a number of aliphatic chloro compounds and concluded that chloride is liberated nonenzymatically by interaction of the halogen compound with SH compounds. However, these findings do not negate those of Heppel & Porterfield since the two groups investigated different halogen compounds.

A typical example of dehydrochlorination is the conversion of DDT to DDE 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene. The enzyme system has been partially purified from DDT-resistant houseflies and requires GSH (Sternburg *et al.* (113)). It is virtually absent in strains of DDT-sensitive flies.

Jondorf *et al.* (114) have shown that rabbits metabolize  $C^{14}$  hexachloroethane to a number of compounds, including tetrachloroethane, tetrachloroethylene, trichloroethanol, trichloroacetic acid, dichloroethanol, dichloroacetic acid, monochloroacetic acid and oxalic acid. The variety of products indicates that other pathways for removing halogen may be present in mammals.

## REPLACEMENT OF SULFUR BY OXYGEN

The replacement of a sulfur by an oxygen atom is an unusual type of biotransformation. Thiobarbiturates are metabolized not only by oxidation of the alkyl sidechain, but are also converted in part to the corresponding oxygen analogues [Raventos (115)]. For example, considerable amounts of thiophenobarbital are converted to phenobarbital in the rabbit:

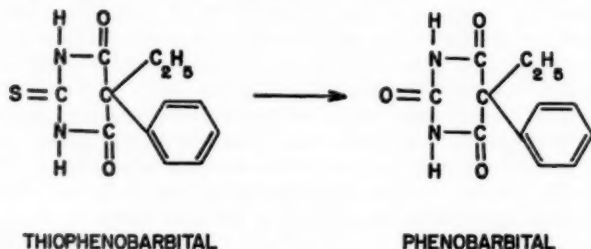
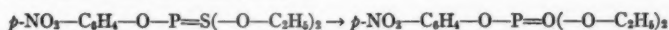


FIG. 5

The loss of sulfur accounts for the radioactivity of  $S^{35}$  urinary inorganic and ethereal sulfate after the administration of thiopental [Taylor *et al.* (116)]. Winters *et al.* (117) have demonstrated that thiopental yields its oxygen analogue pentobarbital in a minced preparation of rat liver. With a similar preparation of rat liver Spector (118) has shown that thioamylal forms the oxygen analogue secobarbital and that thiouracil forms uracil.

Another example of this reaction is the conversion of the pharmacologically inactive parathion, an insecticide, to the potent choline esterase inhibitor paraoxon [Davison (119)].



The enzyme system responsible for this reaction is present in rat liver homogenates, and requires DPN, oxygen, nicotinamide and  $\text{Mg}^{++}$ . The activity is located in the fraction containing microsomes plus the soluble fraction of the cell. The same liver enzyme system may be responsible for the conversion of a  $\text{P=O}$  to a  $\text{P=S}$  moiety in the *in vitro* metabolism of the dimethoxyester of benzotriazine dithiophosphoric acid [Murphy & DuBois (120)].

#### COMPARATIVE METABOLISM OF FOREIGN COMPOUNDS

*Oxidative metabolism of drugs in various animals.*—Gaudette *et al.* (121) have studied the fate of representative drugs metabolized in mammalian liver microsomes in a variety of lower forms of animals. Four species of fish lack the ability to oxidize monomethyl-4-aminoantipyrine, aminopyrine, methyl-aniline, antipyrine, hexobarbital or *p*-ethoxyacetanilide. These compounds are excreted unchanged presumably through the gills. Certain types of amphibia including frogs (*Rana pipiens* and *Rana esculenta*) and salamanders also failed to metabolize these compounds, but excrete them unchanged through the skin. Apparently, the fish gills and the amphibian skin act as lipid membranes since the drugs have been found to penetrate into the animals from the surrounding fluid. Experiments with isolated liver microsomes of fish and amphibia showed that they lack mechanisms for demethylation of monomethyl-4-aminoantipyrine.

Reptiles including alligators and tortoises metabolize the above drugs. The microsomes of these animals contain an enzyme system for demethylating monomethyl-4-aminoantipyrine that requires both TPNH and oxygen. A dealkylating enzyme system with the same requirements is also present in the liver microsomes of birds (chicken, pigeon), the opossum and a large variety of other mammals. Toads (*Bufo marinus*), unlike frogs, have skins that are not permeable to drugs. These amphibia metabolize chlorpromazine, hexobarbital, thiopental, amphetamine, monomethyl-4-aminoantipyrine, aminopyrine and antipyrine. Aminopyrine, antipyrine, and chlorpromazine appear to be metabolized in toad liver homogenates by mechanisms different from those in mammals (121).

*Comparative conjugation of phenols.*—Smith (122) has pointed out that

insects form glucosides rather than glucuronides of phenols but that they do form ethereal sulfates. Maickel *et al.* (123) have studied the comparative conjugation of the phenols, phenolphthalein,  $\alpha$ -naphthol, *p*-nitrophenol and 8-hydroxyquinoline. Several species of fish are unable to conjugate the phenols *in vivo* or *in vitro*. As a result these phenols are highly toxic to fish. In contrast the phenols are readily conjugated by frogs; about 85 per cent of the compounds forming the glucuronide and 15 per cent the ethereal sulfates. In frogs starved for 2 to 3 days, the phenols are conjugated mainly as the ethereal sulfates. The administration of glucose to the frog rapidly restores glucuronide formation as the predominant pathway. Toads also form glucuronides of the above-mentioned phenols. It is of interest, however, that tadpoles of neither toad nor frog can conjugate these phenols.

#### NORMAL ROLE OF ENZYMES INVOLVED IN METABOLISM OF FOREIGN COMPOUNDS

The question often arises as to the normal role of the systems involved in the metabolism of drugs. It is possible that some foreign compounds are converted by enzymes that act essentially on normally occurring substrates while others utilize enzyme systems whose primary function is to act on foreign compounds. Some speculations on the various possibilities are considered below.

*Relatively specific enzyme systems involved in intermediary metabolism.*—The fate of compounds with structures closely related to normal body substrates, the so-called antimetabolites, have not been considered here. Antimetabolites are generally highly polar and are frequently attacked by enzyme systems which act on normal body substrates. For example, the same enzyme deaminates 8-azaguanine and guanine to the corresponding xanthines (124), and 6-mercaptopurine is converted to 6-thiouric acid by the action of xanthine oxidase (125). Antimetabolites may replace the normal substrate in a synthetic reaction. Two examples will suffice: 8-azaguanine and 6-mercaptopurine utilize the same enzyme systems as those of normal purines to form pseudo deoxyribosides in the presence of purine nucleotide phosphorylase (126, 127). Recently, Zatman, *et al.* (128, 129) have demonstrated that isonicotinic acid hydrazide (INH) exchanges with nicotinamide to form the INH analogue of DPN. It is likely that many of the antimetabolites owe their toxic effects to the formation of metabolic products that interfere with normal function; for example, fluoroacetate is thought to be toxic because of its conversion to fluorocitrate [Peters *et al.* (130)].

*Relatively nonspecific enzyme systems which may be involved in both intermediary metabolism and drug metabolism.*—Aldehyde dehydrogenase not only oxidizes foreign aldehydes (51) but also those arising from the oxidative deamination of normally occurring substrates such as norepinephrine, epinephrine, and serotonin (52). The non-specific UDPGA transferase catalyzes the conjugation of such normally occurring substrates as bilirubin (131), estradiol, and testosterone (70), as well as drug substrates. Schmid,

*et al.* (131) found that a mutant strain of Wistar rat which is unable to conjugate bilirubin also shows marked deficiency in its ability to transfer glucuronic acid to menthol and salicylate. The same transferase may be involved in both types of substrates.

*Enzyme systems which may be extremely nonspecific with no role in intermediary metabolism.*—It is intriguing to speculate on the origin of the microsomal oxidative enzymes which are present in land animals but are absent in fish and certain amphibia. Some light is thrown on the question by considering the known changes in the evolutionary transition of animals from sea to land. The problems of conservation of water and consequently the excretion of nontoxic products of metabolism have been vividly discussed by Baldwin (132). In most aquatic animals, the end product of protein metabolism is ammonia which, though toxic, is kept at a safe concentration by its rapid release to the surrounding medium through semipermeable membranes. Land animals cannot excrete ammonia in this manner and instead convert the nitrogen to nontoxic urea or uric acid, excreted through the kidney.

A different problem is the disposal of organic compounds ingested in food which have no role in intermediary metabolism. If lipid soluble, they would accumulate in the body to toxic levels unless there were some way for disposing of them. These compounds readily escape from fish and certain amphibia through the gills and the skin. It is not surprising that these animals do not metabolize a number of foreign compounds.

It is significant therefore that land animals have had to develop enzymes for converting lipid-soluble foreign compounds to more polar substances which the kidney can excrete (*vide infra*).

Ideally, enzyme mechanisms acting on foreign substances should be very nonspecific without depleting the body of essential compounds. The oxidative enzymes in microsomes seem peculiarly well fitted to act selectively on lipid-soluble foreign compounds because they do not catalyze the oxidation of polar substances. In view of the nonspecificity of these microsomal systems, it would not be surprising to find that they catalyzed the oxidation of lipid-soluble normal substrates. Recently Mueller & Rummey (133) have reported that estradiol is oxidized by a microsomal enzyme system requiring TPNH and oxygen. Other examples, especially among the steroids, will probably be found, and it will be of interest to determine whether these reactions are useful or wasteful to the organism.

More comparative studies are needed to decide whether the development of special enzymes for foreign compounds may have been an important factor in making possible the emergence of animals onto land.

#### GENERAL COMMENTS

Numerous reports on the metabolism of foreign organic compounds make it apparent that only a few chemical pathways account for the metabolic conversion of a potentially unlimited number of compounds. The number of



enzyme systems, however, is greater than the number of pathways; for example, there are two or more dealkylation systems in liver microsomes. But the biotransformation of foreign compounds is achieved by enzyme systems with an extraordinary lack of specificity; it is therefore premature and often confusing to name a tissue catalyst after the drug it metabolizes.

Studies with tissue preparations confirm the commonly held view that drug-metabolizing enzymes are present mainly in liver. However, there is little experimental basis for the prevalent opinion that drug metabolism is generally impaired in various liver diseases.

*Action of oxidative microsomal enzymes on normally occurring substrates.*—Traditionally, it is held that foreign compounds undergo chemical transformation because their structures are similar to those of substrates in intermediary metabolism. Examples of this are well known among the anti-metabolites (*vide supra*) but normally occurring substrates are generally not metabolized by microsomal enzymes that oxidize foreign compounds. For example, sarcosine, dimethylglycine, dimethylaminoethanol, choline, creatinine, and epinephrine are not demethylated by microsomes (8), though the demethylation of sarcosine and dimethylglycine is accomplished by highly specific systems in mitochondria (134). Again, L-phenylalanine, L-tryptophan, kynurenine and anthranilic acid are not hydroxylated by microsomes, but by quite specific systems in other parts of the liver cell (24). The inability of microsomes to oxidize these substrates is surprising in view of the unusually broad range of foreign compounds they metabolize. However, a comparison of the rates of metabolism of a series of foreign N-alkylamines has provided some insight into this apparent paradox. Only compounds with a high oil to water partition ratio are metabolized by microsomes *in vitro*, suggesting that the microsomal oxidative systems are protected by a lipid barrier penetrated only by fat-soluble substances (8). Alternatively, the active sites on the microsomal enzymes may permit enzyme-substrate interaction only with nonpolar substances.

*Species and sex differences and their relation to drug metabolism.*—The duration of action of drugs varies greatly in animal species. For example, hexobarbital is metabolized so rapidly in a mouse that a dose of 100 mg./kg. will keep an animal asleep for about 12 min., while 50 mg./kg. will maintain anesthesia in dog or man for over 5 hr. This species variation has been related by Quinn *et al.* (135) to the activity of the liver microsomal enzyme systems. The duration of the response to hexobarbital is proportional to the biologic half-life of the drug and inversely related to the activity of the inactivating enzyme system in liver microsomes (Table I).

Jay (136) has reported marked differences in the duration of action of hexobarbital in various mice strains, but a remarkable uniformity in the response of individual members of the same strain. By contrast, individuals of a nonhomogeneous species like man can show as much as a tenfold variation in the rate of metabolism of certain drugs, e.g., dicumarol (137).

There are also sex differences in response to drugs. Female rats given certain barbiturates sleep considerably longer than do males (138). Quinn

TABLE I

## METABOLISM OF HEXOBARBITAL IN VARIOUS SPECIES

Figures in parentheses refer to number of animals in each species. Dose of barbiturate 100 mg./kg.

	Sleeping time minutes	Hexobarbital half-life minutes	Enzyme activity $\mu\text{g.}/\text{g.}/\text{hr.}$
Mice (12)	$12 \pm 8$	$19 \pm 7$	$598 \pm 187$
Rabbits (9)	$49 \pm 12$	$60 \pm 11$	$294 \pm 28$
Rats (10)	$95 \pm 15$	$139 \pm 54$	$134 \pm 51$
Dogs (5)*	$315 \pm 105$	$261 \pm 20$	$36 \pm 29$

\* Dogs received 50 mg./kg. of hexobarbital.

*et al.* (135) explained this by showing that the livers of male rats have higher microsomal activity than those of females in oxidizing hexobarbital. This difference between the sexes can be altered by sex hormones. Males, pretreated with estradiol for several weeks before hexobarbital administration, sleep just as long as the females; correspondingly, their liver microsomes show a decline in enzyme activity (139). Conversely, female rats pretreated with testosterone react to hexobarbital like male animals; this is reflected by an enhanced enzyme activity in their microsomes (135).

These interesting phenomena apply to rats; but mice, guinea pigs, rabbits and dogs show no sex differences in the metabolism of hexobarbital (138). Furthermore, the sex hormones induce no measurable change in the rates of metabolism of barbiturates in mice and rabbit (139). Why this sex difference is present only in rats is not known.

Other examples of sex difference in the metabolism of drugs have been reported. Axelrod (7) found that the microsomes of male rats are more active than those of females in demethylating various narcotics. In contrast, Davison (119) has made an interesting observation that the insecticide parathion is converted to a toxic derivative more rapidly by female than by male rats.

*Nonspecific inhibitors of drug metabolism.*— $\beta$ -Diethylaminoethyl diphenylpropylacetate (SKF 525-A) has little direct pharmacologic activity of its own but prolongs the action of a variety of drugs by inhibiting their rate of metabolism (140). Its mechanism of action is still not known. It blocks a number of microsomal oxidative reactions including N-demethylation, side-chain oxidation, deamination, hydroxylation and ether cleavage (141). It also inhibits nonoxidative microsomal reactions, for example, the formation of morphine glucuronides (141) and the de-esterification of procaine (139). Furthermore it blocks the function of a soluble enzyme, nitro reductase (54), indicating that the action of SKF 525-A is not a physical effect on microsomes, but is presumably a true enzyme inhibition.

La Du *et al.* (142) showed that the inhibitory action of SKF 525-A on the dealkylation of monomethyl-4-aminoantipyrine is unaffected by the concentration of substrate. They determined some structural requirements in a series of SKF 525-A analogues and demonstrated that the propyl group is needed for maximal activity, but Fouts & Brodie (143, 144) have shown that Lilly 18947 and iproniazid compounds structurally unrelated to SKF 525-A, block the same enzyme systems.

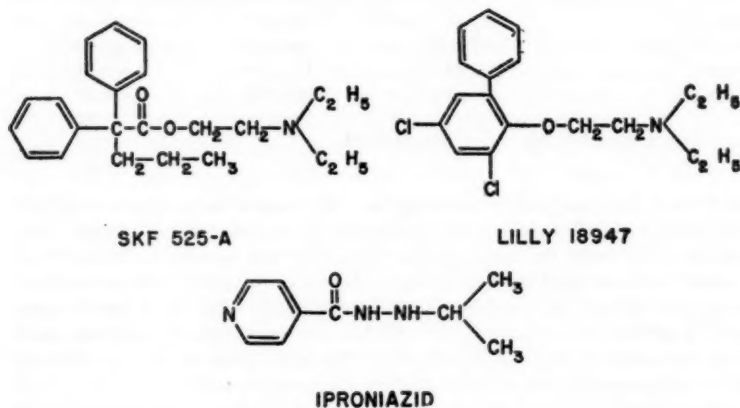


FIG. 6

SKF 525-A has little effect on a number of enzyme systems important in intermediary metabolism including TPN-cytochrome-*c* reductase, glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, phenylalanine hydroxylase and the system that converts tyrosine to fumaric acid (141). In general, the inhibitor has a predilection for enzyme systems that metabolize foreign compounds and catalyze pathways as diverse as oxidation, reduction, and conjugation.

*Activation of drug metabolism by polycyclic hydrocarbons.*—3,4-Benzopyrene, 3-methylcholanthrene and certain other polycyclic hydrocarbons given to weanling male rats, induce a rapid increase in the ability of liver microsomes to oxidize certain foreign compounds [Conney, *et al.* (26)]. Some, but not all of the hydrocarbons are carcinogens, although they all induce increased liver growth. The increased enzyme activity is evident before the increase in liver size; within 6 hr. methylcholanthrene enhances the ability of microsomes to demethylate dimethylaminoazobenzene and to hydroxylate 3,4-benzopyrene. Within 2 hr., the activity of the demethylase increases fivefold, the hydroxylase tenfold and reductive splitting of the azo dye twofold.

The polycyclic hydrocarbons do not provoke an increase in the enzyme activity when they are incubated with liver preparations *in vitro*. The administration of ethionine to the rats completely inhibits the increase in enzyme activity, suggesting the hydrocarbons induce the synthesis of new enzyme activity and do not affect stimulatory or inhibitory factors. The authors have suggested that the hydrocarbons trigger a mechanism leading to increased protein synthesis. The response is partially selective so that certain enzyme activities increase faster and to a greater extent than does the total liver protein concentration. The selectivity of the response for the enzymes that metabolize foreign compounds is emphasized by the finding that the activities of other microsomal enzymes such as glucose-6-phosphatase, TPN-cytochrome-*c* reductase and DPN-cytochrome-*c* reductase are not altered following administration of methylcholanthrene (26). More recently, Takemori & Mannering (145) reported that pretreatment of rats with 3-methylcholanthrene induced no change in N- or O-demethylation of morphine, meperidine, methadone and a number of morphinan derivatives but produced a threefold increase in the capacity to demethylate 3-methyl-4-monomethylaminoazobenzene.

*Role of ascorbic acid in drug metabolism.*—Ascorbic acid may have a role in the metabolism of drugs. Axelrod *et al.* (146) demonstrated that scorbutic guinea pigs were much less efficient in hydroxylating antipyrine and acetanilide than normal animals. King and his associates showed that many drugs increase markedly the urinary excretion of L-ascorbic acid in rats [Longenecker *et al.* (147)]. Burns *et al.* (72) have shown that this effect is not directly related to drug metabolism since barbital, which is not metabolized, induces a sharp increase in the excretion of the vitamin. These drugs exert their effect by stimulating the formation of L-ascorbic acid from glucose via D-glucuronic acid and L-gulonic acid. Just how drugs divert glucose metabolism in this direction and the part played in drug action is not clear.

*Relation between urinary excretion and metabolism of foreign compounds.*—A recent review on physico-chemical factors in drug action pointed out that most drugs are lipid soluble and are passively reabsorbed through the lipoidal membranes that line the renal tubules (148). For example, thiopental is reabsorbed almost completely through the renal tubules, and only about 0.3 per cent of the drug is excreted unchanged (34). Drugs must therefore undergo metabolic conversion to strongly ionized or highly water-soluble derivatives before they can be excreted in appreciable amounts. The so-called "detoxication" mechanisms can therefore be better described as mechanisms to make foreign compounds more polar.

Though most foreign compounds undergo metabolic conversion, certain drugs are excreted virtually unchanged. These are usually strongly ionized compounds like priscoline and hexamethonium, which are chemically unreactive. They are rapidly excreted because their lipid insolubility precludes extensive reabsorption by renal tubules (148). Other biochemically unreactive drugs like ethyl ether or cyclopropane are lipid soluble but are excreted

by the lungs. Barbitol (diethylbarbituric acid) is an interesting example of a drug that is not metabolized in the body, but its lipide solubility is sufficient for extensive reabsorption by renal tubules. As a result the sedative remains in the body for a considerable period of time.

A drug metabolite is almost invariably more polar<sup>5</sup> and therefore less toxic than the parent compound because its decreased lipide solubility prevents it from passing cellular barriers and reaching a potential site of action. However, despite its increased polarity, the derived product may have a pharmacologic action which the parent compound lacks if the conversion should unmask or produce a new functional group. Thus, prontosil is inactive, but is converted to sulfanilamide which is active due to the unmasking of the *p*-amino group. The insecticide parathion is changed *in vivo* to the potent cholinesterase inhibitor by the conversion of a P=S to P=O group. A number of other examples of a similar nature have been cited (148).

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<sup>5</sup> Exceptions to this rule are rare; an interesting one is the reduction *in vivo* of chloral hydrate (the hydrate of trichloroacetaldehyde) to trichlorethanol, which is more lipide soluble than the parent compound.

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## BIOLOGICAL OXIDATIONS<sup>1,2</sup>

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The subject matter will be arbitrarily divided into five broad categories, i.e. Photosynthesis, Respiratory Coenzymes and Enzymes, Oxygen Metabolism, Oxidative Phosphorylation, and Miscellaneous Topics. [Such a classification has many obvious shortcomings but, since a summary to this article is not provided, some busy biochemist may thereby be spared the sad fate of reading the entire review.]

The topic of fatty acid oxidation was thoroughly covered in 1957 (1) and hence it has not been treated again here. Similarly, the substantial work on succinic dehydrogenase has been comprehensively reviewed by Singer *et al.* (2).

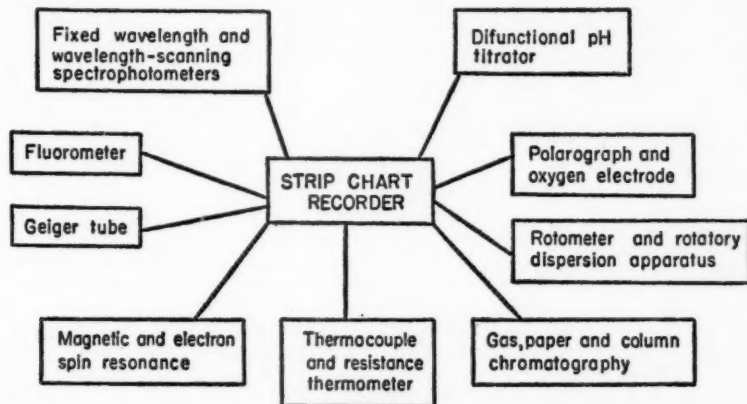


FIG. 1. Some applications of the strip chart recorder in biochemical instrumentation.

We may ponder for a moment on factors responsible for the current profusion of papers dealing with some aspect of biological oxidation. One factor

<sup>1</sup> This review was completed in November, 1957.

<sup>2</sup> The following abbreviations will be used: AMP for adenosine-5'-monophosphate; ADP for adenosine-5'-diphosphate; ATP for adenosine-5'-triphosphate; CoA for coenzyme A; DPN for oxidized diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; FMN for riboflavin-5'-phosphate; FAD for flavinadenine dinucleotide;  $\Delta F'_{\text{hydrolysis}}$  for apparent free energy change in hydrolysis; Pi for inorganic phosphate; PP for pyrophosphate; TPN for oxidized triphosphopyridine nucleotide; and TPNH for reduced triphosphopyridine nucleotide.

is the recent invasion of the biochemical laboratory by the strip chart recorder. Figure 1 illustrates some of the biochemically useful instruments and procedures which may be readily adapted to feed an electrical signal to a potentiometric recorder. The latter is capable of recording any variable which can be transduced to a change in voltage, current or resistance. It is fortunate indeed that in biological oxidation the process of electron transfer can often be detected by a spectrophotometer or other device which may be connected to the strip chart recorder without an elaborate instrumental arrangement.

### PHOTOSYNTHESIS

Recent progress in photosynthetic phosphorylation has been reviewed by Arnon *et al.* (3). The authors point out that photosynthetic and mitochondrial phosphorylations can be readily distinguished on the basis of their oxygen requirements. In the former case phosphorylation proceeds rapidly under strictly anaerobic conditions while in the latter, the reaction is entirely dependent on oxygen. A similar underlying basis is thus suggested for plant and microbial photosynthesis. As a general scheme for photosynthesis in chloroplasts the authors suggest the reaction shown in Figure 2.

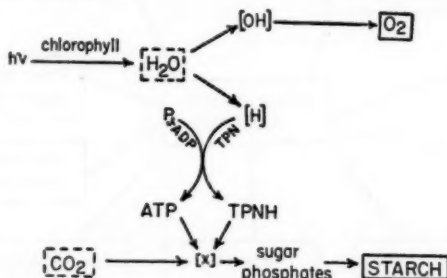


FIG. 2. General scheme of photosynthesis in chloroplasts [Arnon *et al.* (3)].

The ATP and TPNH are needed for the "assimilatory power" required for conversion of  $CO_2$  to sugar. TPN appears to be the specific pyridine nucleotide required and, in view of the insensitivity to  $10^{-3}$  M. arsenite, a role of lipoic acid in the reduction of this coenzyme appears unlikely. The roles of vitamin K, FMN and ascorbate in photosynthetic phosphorylation are discussed (see Figure 3), and Arnon *et al.* (3) conclude:

It is an interesting aspect of biochemical evolution that green plants, after evolving a mechanism independent of molecular oxygen for generating ATP in light, have also shared with non-green organisms the emergence of an oxygen-dependent generation of ATP by phosphorylation.

Avron & Jagendorf (4) described a soluble heat-labile factor from chloro-

plants which markedly increases photosynthetic phosphorylation. Allen *et al.* (5) report the phosphorylation of up to 500 micromoles of orthophosphate esterified per hour per mg. of chlorophyll with isolated chloroplast fragments. These rates are similar to maximum rates of photosynthesis in intact leaves of land plants with optimal light and  $\text{CO}_2$  supply and are on a nitrogen basis several times higher than rates of oxidative phosphorylation from mitochondria from various plant and animal sources.

Wessels (6) has presented evidence for separate pathways of photosynthetic phosphorylation involving menadione and flavin mononucleotide, respectively. Cytochrome-*c* stimulated anaerobic phosphorylation in chloroplasts only in the presence of the former cofactor.

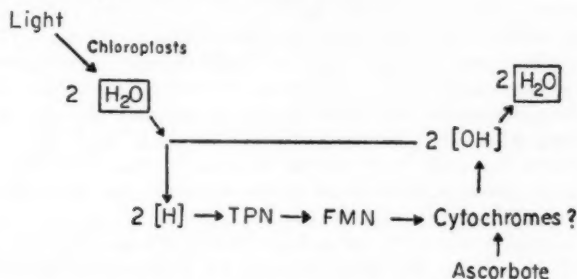


FIG. 3. Role of cofactors in chloroplast phosphorylation [Arnon *et al.* (3); Wessels (6)].

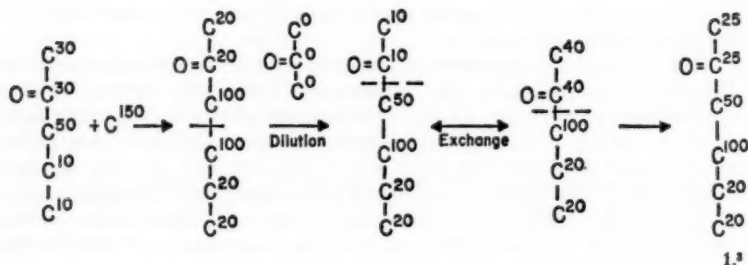
The association of vitamin K with respiratory chain phosphorylation, previously reported by Martius & Nitz-Litzow (7) has been confirmed by Dallam & Anderson (8). According to Brodie *et al.* (9) the evidence that vitamin K plays an essential role in a physiological electron carrier in oxidative phosphorylation in *Mycobacterium phlei* can be summarized as follows: (a) restoration of oxidative phosphorylation in light-inactivated preparations by the addition of vitamin  $\text{K}_1$ , (b) the reconstitution of coupled phosphorylation by adding  $\text{K}_1$  after extensive fractionation of the supernatant, and (c) the specific requirements for certain configurations which occur in vitamin  $\text{K}_1$ , but not in related compounds.

From a study of the effect of inorganic iron deficiency in green algae, Kessler (10) has concluded that manganese is specifically required for the oxygen release mechanism. Lack of iron decreases photoreduction while lack of phosphate affects both photosynthesis and photoreduction. Thus phosphate is associated with reduction of  $\text{CO}_2$  and iron with the hydrogenase system. San Pietro & Lang (11) observed an incorporation of deuterium from the medium into DPN or TPN of illuminated grana. The most likely mechanism to account for this phenomenon would be reduction and reoxidation by enzymes with dissimilar stereospecificity for the pyridine ring.

A number of interesting experiments have been carried out in the area of

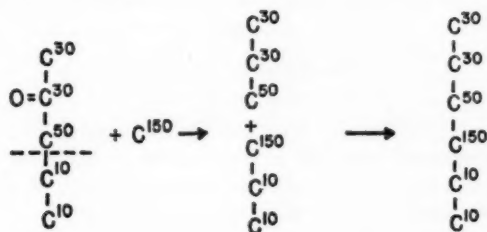
the physics and chemistry of photosynthesis. Fujimori & Livingston (12) report the reaction of triplet state chlorophyll with oxygen, carotene and other substances. Both  $\beta$ - and  $\alpha$ -carotene as well as xanthophyll were as efficient as oxygen in quenching the triplet state of chlorophyll. This strengthens the suspicion [see Lynch & French (13)] that carotene plays a direct role in the primary photosynthetic act. Arnold & Sherwood (14) have examined the possibility that chloroplasts act as semiconductors. With *Chlorella* suspensions, leaves and dried chloroplasts, glow curves can be obtained analogous to those made with inorganic crystals. They conclude that "the first act in photosynthesis may have as much in common with steady-state physics as with the chemistry of solutions." The long wave-limit of photosynthesis has been studied by Emerson *et al.* (15) while Sogo *et al.* (16) have verified and extended the findings of Commoner *et al.* (17) on the electron spin resonance obtained from light-induced paramagnetism in chloroplast suspensions. Duysens & Sweep (18) prefer fluorescence to absorption spectrophotometry for investigation of pigment changes in photosynthesizing cells. The difference between the "dark" and "light" fluorescence spectra in purple bacteria and blue-green algae resembles that of DPNH and there appears to be an accelerated reduction of pyridine nucleotides during photosynthesis.

The expulsion of exactly one mole of chlorophyll-bound  $\text{CO}_2$  on fluoride treatment of *Chlorella* has been recorded by Warburg & Krippahl (19). Warburg (20) has reviewed all of the photosynthesis work, past and present, from his laboratory, and of particular interest is the demonstration that *Chlorella* cells contain a quantity of loosely bound glutamic acid which can be released into the medium on heating. If, however, the cells are heated after the addition of fluoride, the glutamic acid is replaced by  $\alpha$ -aminobutyric acid. Gibbs & Kandler (21) have considered three mechanisms whereby the labelling pattern in glucose might lead to a ratio for C-4/C-5 or C-6 > C-3/C-1 or C-2. In the first mechanism "cold" dihydroxyacetone phosphate from a pool, derived from metabolism of endogenous fat, dilutes the isotopic metabolite prior to condensation. Subsequent to condensation, a transketolase-



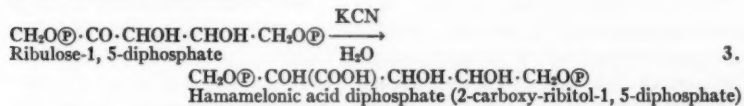
\* Numbers refer to relative radioactivities of the various carbon atoms.

catalyzed exchange of C-1 and C-2 may occur between fructose and pentulose phosphates so as to increase the label in the former molecule. According to the scheme shown in Reaction 1, a combination of dilution by endogenous dihydroxyacetone phosphate and an exchange catalyzed by transketolase could produce a hexose unit with isotope distribution in accord with the observed facts. A second scheme (Reaction 2) proposes a splitting of pentulose phosphate at C-3/C-4 followed by condensation of the diose phosphate with a reduced form of  $\text{CO}_2$ .

2.<sup>1</sup>

The third and final mechanism suggests (a) addition of  $\text{CO}_2$  to C-4 of the pentose (b) reduction of this addition product (c) rearrangement whereby the new carbon becomes C-4 and (d) a lack of reducing power through the formation of two molecules of phosphoglyceric acid from the primary addition product.

In an attractive little book, Bassham & Calvin (22) review the path of carbon in photosynthesis. *Scenedesmus* suspensions have been investigated by Metzner *et al.* (23) with a view to finding the real form of  $\text{CO}_2$  utilized by carboxydismutase. One of the possible sources of error in the use of cyanide as a metabolic poison, alluded to several years ago by Warburg (24), has now been encountered in photosynthesis. For instance, Rabin *et al.* (25) have shown that treatment of algae with high concentrations of cyanide in photosynthesis experiments leads to the formation of hamamelonic acid diphosphate according to Reaction 3.



Saltman *et al.* (26) found incorporation of  $\text{CO}_2$  into the Krebs cycle intermediates (and amino acid analogues) during dark fixation by leaves of *Kalanchoe pinnata* (26), but reported no difference in the labelling pattern in leaves from photo-periodically induced and noninduced *Kalanchoe blossfeldiana* (27).

Photo oxidations catalyzed by FMN have been studied in plant and bacterial extracts by Vernon & Ihnen (28).



## RESPIRATORY COENZYMES AND ENZYMES

## PYRIDINE NUCLEOTIDES AND PYRIDINOPROTEINS

*Stereospecificity.*—Vennesland's group (29) has shown that both muscle and yeast triose phosphate dehydrogenase catalyze a direct hydrogen transfer between substrate and  $\beta$ -4-position of the nicotinamide ring. Table I, taken from a still more recent paper (30), lists the side of the pyridine ring, designated as either  $\alpha$  or  $\beta$ , which is acted on by a number of dehydrogenases. It would obviously be of interest to extend this list, and likely candidates for

TABLE I  
STERIC SPECIFICITY FOR DPN  
[From Levy & Vennesland (30)]

Dehydrogenase	Source	Steric specificity
Alcohol (with ethanol)	Yeast, <i>Pseudomonas</i> , liver, wheat germ	$\alpha$
Alcohol (with isopropyl alcohol)	Yeast	$\alpha$
Acetaldehyde	Liver	$\alpha$
L-Lactate	Heart muscle	$\alpha$
L-Malate	Pig heart, wheat germ	$\alpha$
D-Glycerate*	Spinach	$\alpha$
Dihydroorotate	<i>Zymobacterium oroticum</i>	$\alpha$
$\alpha$ -Glycerophosphate	Muscle	$\beta$
3-Phosphoglycerinaldehyde	Yeast, muscle	$\beta$
L-Glutamate	Liver	$\beta$
D-Glucose	Liver	$\beta$
$\beta$ -Hydroxysteroid	<i>Pseudomonas</i>	$\beta$
DPNH cytochrome-c†	Rat liver mitochondria, pig heart	$\beta$
TPNH (transhydrogenase)‡	<i>Pseudomonas</i>	$\beta$

\* Unpublished experiments with F. A. Loewus and H. A. Stafford.

† Drysdale & Cohn (31).

‡ San Pietro *et al.* (32).

investigation should include the pyridine nucleotide-linked lactic dehydrogenases from both D(−) and L(+)-forming homolactic bacteria. At the present time there appears to be no rational basis for predicting which type of specificity is to be expected. Thus there is no apparent correlation with any structural feature or chemical property of the substrate or enzyme nor is there any seeming connection between the steric specificity and the kinetic characteristics of the catalyzed reaction. Be this as it may, the existence of stereospecificity provides, as Levy & Vennesland (30) have pointed out, a subtle mechanism for selectivity between metabolic pathways involving protein-bound pyridine nucleotides. In such cases, hydrogen transfer between complexes with opposite specificities would be expected to proceed with a maximum rate.

*Analysis of pyridine nucleotides and related problems.*—A number of improvements have recently been introduced for the laboratory manipulation of the pyridine nucleotide coenzymes. The fluorometric determination of pyridine nucleotide coenzymes has been carefully investigated by Lowry *et al.* (33). Procedures were worked out for measurement of low concentrations of the oxidized or reduced form in the presence of large amounts of the opposite oxidation level of the coenzyme and the utility of the method has been illustrated by measurement of the kinetic constants of certain pyridine nucleotide dehydrogenases. Fluorescence spectrophotometry has been used by Duysens & Ames (34) for determination of DPN *in vitro* and *in vivo*. This particular technique, although perhaps not as convenient, appears to be more specific for the pyridine nucleotides than the more commonly used absorption spectrophotometry. For example, Duysens & Sweep (18) showed that irradiation of suspensions of purple bacteria and of blue-green algae with photosynthetically active infrared or red radiation caused an increase in blue fluorescence. The difference spectra for dark vs. light fluorescence resembled that of reduced pyridine nucleotide, indicating an accelerated reduction of the coenzyme(s) during photosyntheses. Interference by other pigments was not serious.

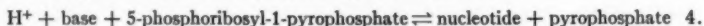
A number of investigators have measured the ratio of oxidized to reduced pyridine nucleotide in living systems. Thus Holzer *et al.* (35) found the quotient DPNH/DPN in suspensions of living yeast cells to have the following values—Anaerobic conditions with glucose, 1/400; aerobic conditions with glucose, 1/1100. The latter ratio became 1/3000 and 1/3600 with added ammonium ion and 2,4 dinitrophenol, respectively. In a comparison of fatty and normal liver, the former was shown to contain 10 per cent less total pyridine nucleotide than normal liver and the ratio DPN/DPNH is 2:1 as opposed to 3:1 (36).

The *in situ* behavior of the pyridine nucleotides in tissues has continued to command attention. McIlwain & Tresize (37) maintain that the concentrations of reduced coenzymes and of glutathione in cerebral tissues approach but do not exceed the levels required for maximum rate of oxidation. The use of tetrazolium ion has permitted the detection of dehydrogenase activity in capillaries of the human central nervous system, spleen, heart, bone marrow and some endocrine glands. Only very slight activity was found in capillaries from liver, kidney and anterior pituitary and none at all in lung (38). The lactic dehydrogenase of normal serum has been found distributed among three electrophoretically distinct fractions (39). Human red cell hemolysate showed similar pattern and the possibility was considered that the three active peaks might represent lactic dehydrogenase from different sites of origin. The activity of both lactic dehydrogenase and phosphohexoisomerase is apparently much greater in perilymph than in cerebro-spinal fluid or serum (40).

A method for production of reduced pyridine nucleotides by controlled potential cathodic reduction has been devised by Powning & Kratzing (41).

*Metabolic reactions of pyridine nucleotides.*—A number of excellent papers

dealing with the biosynthesis and metabolism of the pyridine nucleotides have appeared. Preiss & Handler (42), working with human erythrocytes *in vitro*, showed that nicotinic acid, but not the amide, served as an effective precursor of DPN. It was concluded that free nicotinamide is not an intermediate in DPN synthesis from nicotinic acid; i.e., amidation may occur after the pyridine ring has been attached to a nucleotide derivative. These authors (43) report the syntheses of nicotinamide mononucleotide and a number of purine nucleotides, according to Reaction 4:



The 5-phosphoribosyl-1-pyrophosphate was obtained by reacting ATP with ribose-5-phosphate and the specific enzyme synthesizing nicotinamide mononucleotide was purified six hundred fold. The nicotinic acid analogue of DPN, deamido DPN, has now been obtained by isolation from *P. chrysogenum* and by incubation of nicotinic acid with human erythrocytes (44).

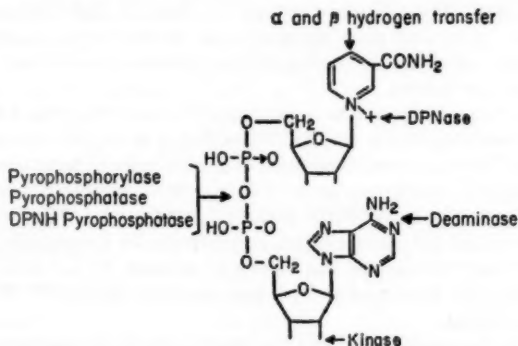


FIG. 4. Some enzymatic transformations of diphosphopyridine nucleotide.

The conversion of a number of pyridine derivatives to DPN has been studied by Kaplan *et al.* (45) and the possibility has also been considered that these substances are converted to nicotinamide at the glycosidic level.

The properties of a reduced pyridine nucleotide pyrophosphatase from pigeon liver have been charted by Jacobson & Kaplan (46). The products of the reaction of DPN were shown to be reduced nicotinamide mononucleotide and 5'-adenylic acid. The enzyme also split FAD, adenosine diphosphate ribose and TPNH but did not attack the normal oxidized forms of either pyridine nucleotide coenzyme. The attractive hypothesis is presented that the function of the enzyme may be to hydrolyze the metabolically useless adenosine diphosphate ribose arising from the action of the enzyme DPNase. The distribution of DPN pyrophosphatase, DPNH pyrophosphatase and DPN nucleotidase has been measured in cellular fractions from several organs of the hamster, mouse, pigeon, and rat (47). Microsomes from liver,

kidney, and brain yielded the highest activities of DPN pyrophosphatase and DPN nucleotidase while the soluble fraction of pigeon and rabbit livers contained one half of the total DPNH pyrophosphatase. In other organs the major concentration of DPNH pyrophosphatase was found in the microsomes. Nucleosidases from erythrocytes of different species may be differentiated according to their specificity for DPN or TPN (48). Some of the known enzymatic transformations of DPN are shown in Figure 4. In order to obtain information on the nature of the protein-linked groups in pyridine nucleotides, Astrachan *et al.* (49) have made a thorough study of the enzymatic reactions of the bound coenzymes. They showed that DPN nucleotidase from *N. crassa* splits the coenzyme bound to inactive triose phosphate dehydrogenase. In the case of active triose phosphate dehydrogenase, the bound coenzyme is also split if the pH is outside the range 7.4 to 8.0 DPN is deaminated slowly in the inactive enzyme and not at all in the active enzyme by takadiastase. However, DPNH bound to active triose phosphate dehydrogenase is deaminated at a rate comparable to that found with free DPNH. DPN kinase is able to more readily phosphorylate the coenzyme bound to the inactive protein. Similarly, the DPN of inactive triose phosphate dehydrogenase, in contrast to the active enzyme, is available for interaction with a number of dehydrogenases. It was concluded that in the active enzyme, DPN is bound through the nicotinamide moiety and through at least one other linkage.

Rafter & Colowick (50) describe the formation of the elusive DPNH derivative, DPNH-X, in a system containing pyrophosphate, acetyl phosphate, DPNH and triose phosphate dehydrogenase. Pyrophosphate inhibited the phosphatase activity of the dehydrogenase.

*Mechanism of action.*—Although a detailed understanding of the mechanism of action of any enzyme is still lacking, it can be predicted with confidence that a solution to this problem will soon be achieved. Particularly impressive in this connection is the work with chymotrypsin (51) and the intriguing possibility that a similar active center may occur in other enzymes such as phosphoglucomutase (52). Since the respiratory enzyme always occurs inside the cell and is situated at the seat of metabolism, it would appear to be a somewhat more fundamental ingredient of life processes than the proteolytic enzyme. The mechanism of action of the respiratory enzymes is thus a prime importance for the understanding of the chemistry of life.

From a study of the isotope rate effect, Mahler & Douglas (53) conclude that yeast alcohol dehydrogenase can bind coenzyme and substrate independently. A transition state analogous to that found in the Meerwein-Ponndorf-Oppenauer reaction was postulated. Similar conclusions were reached in an almost simultaneous series of papers appearing from Wallenfels' laboratory. In the first paper, they report a simple method for crystallization of alcohol dehydrogenase from yeast (54). The enzyme contained 36 free SH groups, each of equal reactivity. Heavy metal inhibition could be reversed with glutathione and zinc. The nondialyzable zinc content (of "zinc

enzyme") varied from 35 to 5 atoms per mole (150,000 g.), the lower levels of metal ion still representing fully active enzyme. At higher DPN concentrations the reaction velocity was markedly increased by zinc (55). In an attempt to set up an enzyme model, mixed sulfur and nitrogen-containing zinc chelates were prepared with orthophenanthroline, adenine and adenosine as nitrogen ligands and thiophenol, 3,4-dithioltoluene and 2,3-dithiotartaric acid as sulfur-containing ligands. In these models the nitrogen and sulfur ligands are supposed to represent the pyridine nucleotide and apodehydrogenase, re-

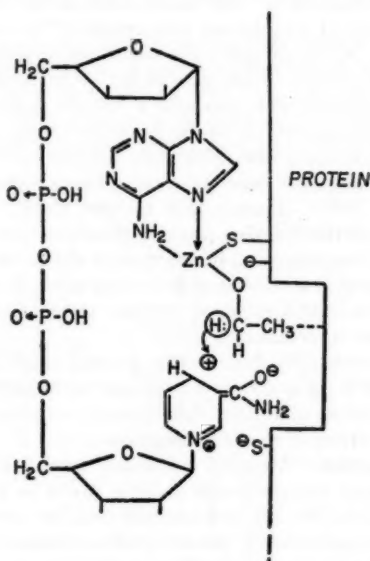


FIG. 5. Enzyme-coenzyme-substrate compound of yeast alcohol dehydrogenase according to Wallenfels & Sund (58).

spectively (56). A simple method of crystallization of glutamic dehydrogenase was devised and it was discovered that the inhibition power of a number of complexing agents on both the alcohol and glutamic dehydrogenases was in agreement with the binding constants of the inhibitors for zinc (57). Ultracentrifugation studies revealed the presence of about 5 and 23 DPN-binding sites per mole of alcohol and zinc-alcohol dehydrogenase, respectively. All of the data obtained for alcohol dehydrogenase lead to the following "map" of the active site (Fig. 5) (58): The reaction mechanism for the system acetaldehyde, DPNH, and alcohol dehydrogenase was considered to resemble the Meerwein-Ponndorf-Oppenauer reduction with the aluminum replaced by zinc-protein in the case of the enzymatic catalysis. [See Mahler &

Douglas (53).] The formation of adducts between two moles of dihydropyridine derivatives and sulfurous acid was examined and similar complexes were proposed as intermediates in the enzymatic reduction of keto acids by reduced pyridine nucleotides (59). A more detailed paper has recently appeared from Vallee's laboratory (60) dealing with the function of the zinc in the alcohol dehydrogenase. During purification of the horse liver enzyme, the zinc content rises to the maximum value of 2 atoms per mole. This evidence plus the fact that zinc-binding agents inhibit the activity, leads to the conclusion that the zinc is an active constituent of the enzyme.

A number of pyridine bases and analogues of DPN were found by Van Eys & Kaplan (61) to compete with the coenzyme for the active site of yeast alcohol dehydrogenase. They found four coenzyme-binding sites per mole (62) of which only one at a time appears to be involved in the activity. Evidence has been obtained that complex formation between pyridine coenzyme, enzyme, and a nucleophilic compound structurally related to the substrate may be a general reaction for dehydrogenases oxidizing an hydroxyl to a carbonyl (63). In the case of yeast alcohol dehydrogenase, the specificity of the reaction would appear to depend on (a) the nucleophilic character, (b) the molecular dimensions and (c) the orientation of the alcohol molecule (64).

In leaves of *Mentha piperata*, an enzyme is present which oxidizes a number of primary and secondary alcohols as well as the cyclic alcohol menthol (65).

A number of authors have surveyed the action of the available inhibitors, particularly the thiol reagents, in order to gain greater insight into the structure and function of the dehydrogenases. Yeast and muscle triose phosphate bind 6 and 11 moles of 0-iodosobenzoate, respectively (66). The treated protein exhibited high phosphatase activity toward acyl phosphate but lost the capacity to oxidize glyceraldehyde. The latter activity could, however, be restored with cysteine. Fuchsin competitively inhibited glutamic dehydrogenase acting on glutamic acid (67) whereas silver nitrate inhibited the enzyme competitively with glutamic acid and noncompetitively with DPN (68). Pre-addition of glutamic acid, but not of DPN, protected against silver nitrate inhibition and cysteine partly reactivated the enzyme. The quantitative effect of hydroxylamine, sulfite, and iodoacetate on a number of DPN-dependent enzymes has been measured (69) and both DPN and DPNH may specifically protect sulfhydryl groups in certain dehydrogenases (70). Stoppani & Milstein (71) have also studied the effect of thiol reagents on yeast and liver aldehyde dehydrogenase.

Redetzki & Nowinski (72) have shown that low concentrations of ortho-phenanthroline activate yeast alcohol dehydrogenase through the removal of contaminating heavy metal ions found in the usual laboratory reagents. The real turnover number of the enzyme should therefore be increased from about  $30 \times 10^3$  up to  $42.9 \times 10^3$  moles alcohol minute<sup>-1</sup>. Shifting of the apparent equilibrium constant of the alcohol dehydrogenase reaction caused by a

combination of cysteine with the product of the reaction has been reported by Macleod *et al.* (73).

Transhydrogenase of animal tissues has been thoroughly investigated by Humphrey (74). The enzyme, which was localized in the mitochondria, was purified by ammonium sulfate fractionation and shown to have a pH optimum of 6.3. No evidence was found for the presence of a flavin or metal prosthetic group. The enzyme from a particulate preparation from heart muscle has been investigated by Ball & Cooper (75) who found TPNH oxidation in the presence of traces of DPN. The reduced form of the latter appears to be removed via the main oxidative pathway. Thyroxine at low concentrations ( $10^{-8}$ M) caused appreciable inhibition of the transhydrogenase system.

Zelitch (76) finds L(+) lactic acid dehydrogenase to be inhibited by certain hydroxy sulfonates. Sodium bisulfite also inhibits, probably through formation of  $\alpha$ -hydroxy sulfonates with the products of the enzymatic reactions.

Studies with highly purified TPN-linked isocitric dehydrogenase from heart (77, 78) suggest that an enzyme-bound form of oxalosuccinate serves as an intermediate. All of the various activities of the enzyme were retained to an equal degree during purification.

Interest in glutamic dehydrogenase has increased in recent years. Homogenization of mouse and rat liver or kidney tissue in water leads to higher activity of the enzyme than homogenization in phosphate buffer, sodium chloride or potassium chloride solutions of equivalent ionic strength (79). Caughey *et al.* (80) have reported the effect of thyroxine on glutamic dehydrogenase and have assessed the structural requirements for substrate competition in this enzyme. Glutarate and  $\alpha$ -ketoglutarate, unlike certain other dicarboxylic acids, were found to be powerful competitive inhibitors. The same inhibitors were found to block the forward and reverse reaction. Thyroxine appeared to inhibit by a "stoichiometric" or "uncompetitive" titration of the enzyme. Glutamic dehydrogenase has been extracted from both the wild-type and a mutant strain of *N. crassa* and the properties of the two enzymes have been compared (81). They differ in several respects, e.g. the mutant enzyme activated by exposure to 35 to 50° for a few minutes or by incubation with substrates.

The supposedly well-established reaction mechanism for triose phosphate dehydrogenase, in which a hemimercaptal is formed between substrate and thiol group of the enzyme, is not accepted by the Warburg school (82), who have now attempted to carry out the oxidation with the muscle enzyme, containing firmly bound DPN, and glyceraldehyde as substrate. Under these conditions no reaction could be obtained without either added Pi or excess free DPN; the latter was considered to provide inorganic phosphate as an impurity.

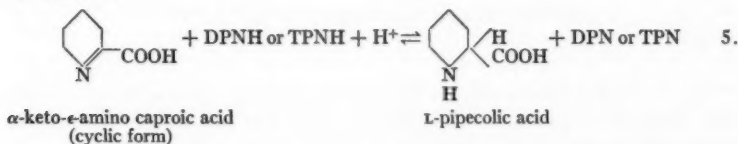
*Miscellaneous reactions.*—The pyridine nucleotides, as the workhorses of



cellular oxidation, are found to catalyze more and more reactions. According to Kun (83) crystalline lactic dehydrogenase and DPNH reduces  $\beta$ -mercapto-pyruvate. A preparation of L-gluconic dehydrogenase from guinea pig liver and kidney contains both TPN and DPN-specific enzymes and DPN-linked D-sorbitol-dehydrogenase (84). With L-gulonic acid as added substrate, the products are D-glucuronic acid and D-xylulose, respectively. In *Aspergillus flavus-oryzae* the DPN-requiring dehydrogenases for glucose-6-phosphate and 6-phosphogluconate, in contrast to the TPN-linked enzymes for these substrates, appear only after the third day of growth. The DPN enzymes are considered as separate and distinct enzymes from the TPN counterparts (85). Fumarate and a number of Krebs cycle intermediates serve as suitable sources of electrons for reduction of the C-20 carbonyl of tetrahydrocortisone and 17-hydroxy-pregnanolone in the presence of a pyridine nucleotide enzyme and a fractionated rat-liver homogenate.

Sanadi & Searls (87) have shown that  $\alpha$  ketoglutaric dehydrogenase catalyzes the reversible oxidation of DPNH by 6,8-lipoic or 6,8-lipoic acid amide.

Meister & Buckley (88) have discovered a pyridine-nucleotide linked enzyme in liver which reduces the keto acid analogue of lysine according to Reaction 5:

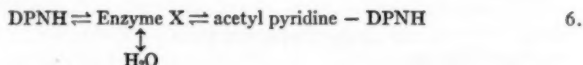


Since several of the pyridine nucleotide apodehydrogenases are obtainable in reasonable quantities in a relatively pure state, an excellent opportunity is thus afforded for immunological experimentation. Crystalline yeast alcohol dehydrogenase from a baker's and brewer's strain of *Saccharomyces cerevisiae* acted as antigens on rabbits and the antienzyme serum inhibits the enzymes (89). Repeatedly recrystallized triose phosphate dehydrogenase from beef and pork does not act as an antigen for rabbits or guinea pigs (90).

#### FLAVOPROTEINS

The elegant work of Beinert on the mechanism of action of flavoprotein acyl dehydrogenases, mentioned briefly in last year's review (1), has now appeared in more detail (91, 92). The specimens studied included the C<sub>4</sub> to C<sub>16</sub> yellow acyl dehydrogenase of pig liver, L-amino acid oxidase of snake venom and the old yellow enzyme. Electron paramagnetic resonance absorption demonstrated the intermediate appearance of a free radical having the typical absorption spectrum of a flavin semiquinone. Recent polarographic measurements also implicate a semiquinone structure during the reduction of flavin nucleotides (93).

Frieden (94), in a careful study of the kinetics of heart DPNH-cytochrome-*c* reductase, has detected three ionizing groups involved in the enzymatic reaction. Two groups are associated with the oxidation of the DPNH, while the third is concerned with the reduction of cytochrome-*c*. A transhydrogenase activity different from that found in *Pseudomonas fluorescens*, has been described by Weber & Kaplan (95). The reaction consists of an electron transfer from reduced pyridine nucleotides to their oxidized acetyl analogues and is catalyzed by typical FAD enzymes but not by FMN enzymes or nonflavin dehydrogenases or reductases. Of some interest is the fact that removal of FAD from the enzymes failed to suppress the activity. Hence, it would seem that electron transfer is mediated by the apoprotein itself. Weber *et al.* (96) have also shown through the use of deuterium tracer that the reduction of acetyl pyridine-DPN by DPNH catalyzed by pig heart diaphorase, proceeds through an electron rather than a direct hydrogen transfer. The reaction sequence is believed to be (a) transfer of an electron to a group (X) on the enzyme forming a reduced protein (b) release of  $H^+$  to make a semireduced DPN and (c) subsequent transfer of an electron to an acceptor. The following scheme (Reaction 6) accounts for these reactions as well as the observed exchange between DPNH or acetyl pyridine DPNH and water—



An FAD diaphorase specific for TPNH has been purified from spinach leaf chloroplasts by Avron & Jagendorf (97). The enzyme reduces several artificial dyes but not cytochrome-*c*. In another interesting development, the diaphorase-like action of a complex of triose phosphate dehydrogenase and 2,6-dichlorophenolindophenol is described (98). The complex catalyzed the oxidation of DPNH by the dye.

DeBernard (99, 100) describes the preparation of a soluble DPNH-cytochrome-*c* reductase from beef heart mitochondria. The enzyme exhibits a modified flavoprotein spectrum and the prosthetic group is believed not to be identical with FAD. A firmly attached heme component and two atoms of iron were present. The DPNH peroxidase of *S. faecalis* has been isolated by Dolin (101) and shown to be a flavoprotein containing 0.66 per cent FAD and no metal or hematin. A DPNH-flavoprotein complex was detected spectrophotometrically. Lyxoflavin nucleotides prepared by Huennekens *et al.* (102), showed some activity with certain apoflavoproteins. Silver (103) describes the nitrate reductase of a nitrate-reducing yeast, *Hansenula anomala*, as a reduced pyridine nucleotide linked metalloflavoprotein with molybdenum as a probable cofactor. According to Peck & Gest (104), the hydrogenase of *C. butylicum* is a ferroflavoprotein showing strong preference for 1 electron dyes. There is also evidence that a second metal, probably molybdenum, may be involved. In cow's milk, a system of enzymes converts

FAD to free riboflavin in a series of steps, the last of which is the dephosphorylation of FMN (105).

The paper chromatographic behavior of flavin compounds has been extensively investigated by Kilgour & Huennekens (106) who also prepared riboflavin-5-pyrophosphate by phosphorylation of FMN with inorganic phosphate and di-*p*-tolylcarbodiimide (107). Boulanger *et al.* (108) have employed L-amino acid dehydrogenase of liver for determination of  $\alpha$ -labelled N<sup>15</sup> ornithine and lysine.

The microsomal cytochrome reductase of calf liver has been isolated in essentially homogeneous form by Strittmatter & Velick (109). The prosthetic group is FAD and approximately two atoms of Mg<sup>++</sup> per mole of flavin are present.

#### CYTOCHROMES

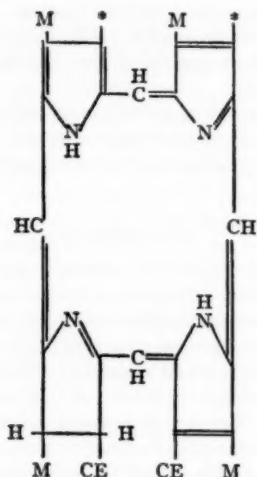
*General properties.*—An alternate method of purification of cytochrome-*c* has been suggested in which the enzyme is extracted from horse heart with dilute H<sub>2</sub>SO<sub>4</sub>, adsorbed on permutite and eluted with dilute NH<sub>4</sub>OH (110). Losses of activity in cytochrome-*c* occur during certain purification procedures and hence a biochemical standardization of the enzyme on liver homogenate or succinic dehydrogenase has been suggested (111).

To the elegant series of papers from Okunuki's laboratory on the crystallization and characterization of cytochrome-*c*'s must be added a report dealing with the N-terminal amino acid of the enzyme (112). Various chromatographically separable fractions of crystalline yeast cytochrome-*c* each contained two N-terminal residues, namely, threonine, and arginine. The crystalline enzyme from either horse or beef heart contained only arginine as the N-terminal amino acid. Paléus & Theorell (113) confirmed the procedures of the Japanese workers for obtaining crystalline cytochrome-*c* from beef heart but the product exhibited three peaks in the electrophoresis apparatus.

The biosyntheses of cytochrome-*c* has recently been extensively investigated by Drabkin and co-workers. Glycine and lysine labelled in the alpha carbon with C<sup>14</sup> were used as precursors with intact rats and rat tissues. The protein moiety acquired the label sooner than the hemin (114). In work with yeast, Yčas & Drabkin (115) found active incorporation of the glycine alpha carbon into cytochrome-*c* hemin under aerobic conditions. These experiments suggest that all aerobic tissues are capable of the independent syntheses of hemoproteins.

Barrett (116) describes the isolation of a green hemin, very likely that of cytochrome-*a<sub>3</sub>*, from *Aerobacter aerogenes*. Comparison of spectra, study of the side chains and conversion into a porphyrin have led to the conclusion that the molecule is a dihydroporphyrin (chlorin) derived from either protoporphyrin or a similar porphyrin (Formula I). Keilin (117) has found the helicorubin of the gastrointestinal tract of the edible snail, *Helix pomatia*, and a closely related intracellular hemochrome of the same species, *cyto-*

*chrome-h*, to be acidic hemoproteins with molecular weights close to 18,500. Helicorubin probably represents a slightly modified form of cytochrome-*h* liberated during the normal excretory activity of the digestive gland of the snail.



I. The chlorin from cytochrome-*a*<sub>3</sub>, according to Barrett (116). M=Methyl; CE=carboxyethyl; \*=vinyl, or a combination of hydroxyethyl or ethyl with vinyl.

While testing cytochrome-*c* as a Hill reagent in spinach chloroplasts, Nie-man & Vennesland (118) observed rapid reduction of the hemoprotein on illumination. The subsequent addition of digitonin resulted in reoxidation of the cytochrome via a photooxidase.

Wald & Allen (119) report the half-saturation pressure of CO on cytochrome oxidase to be 0.17 mm. at 10 to 13°.

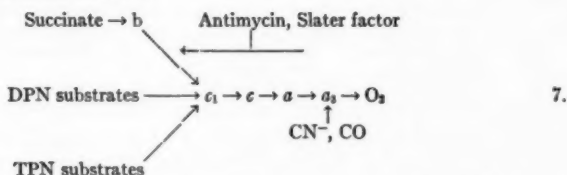
Crane & Glenn (120) have described use of deoxycholate as a reagent for fragmentation of the electron transport particle into red and green components. The red particle contains cytochromes-*b* and -*c*<sub>1</sub> but not -*a*, and catalyzes the oxidation of succinate or DPNH by ferricyanide and cytochrome-*c*. The green particle contains cytochrome-*b*, -*c*<sub>1</sub>, and cytochrome-*a* and catalyzes the oxidation of DPNH via cytochrome-*c*. Antimycin blocks the cytochrome-*c* oxidation of succinate (red particle) and the oxidation of DPNH by either cytochrome-*c* or oxygen (green particle). Mackler & Penn (121) report the separation of DPNH oxidase into a red, antimycin-sensitive particle with DPNH-cytochrome-*c* reductase activity and a green particle with cytochrome oxidase activity. The fragmentation appears to explain the "opening phenomenon" in which DPNH oxidase activity is abolished by deoxycholate and replaced by DPNH-cytochrome-*c* reductase and ferro-cytochrome-*c* oxidase activities. The red particle carries the bulk of the

flavin and all of the cytochrome-*b* and -*c*<sub>1</sub>, whereas the green particle contains all of the cytochrome-*a*. Nonheme iron appears in both fractions but in the green particle copper and heme are concentrated fourfold over the parent particle.

According to Yčas (122) the growth of a normal yeast strain at 40° causes 95 per cent of the colonies to lose cytochromes-*a* and -*c* when plated out at 30°. This deficiency survives through many subsequent transfers at 30°.

Estabrook (123) has described the use of low temperature spectroscopy for sharpening, splitting and intensifying some of the absorption bands of reduced hemoproteins. The utility of the technique in detecting cytochromes-*a*, -*a*<sub>3</sub>, -*b*, -*c*<sub>1</sub> and -*c* in heart muscle is demonstrated (124) and of particular interest is the spectral difference between bound cytochrome-*c* and cytochrome-*c*<sub>1</sub>. In an investigation of the role of these heme-proteins in heart muscle, Ball & Cooper (125) report cytochrome-*c* to restore fully the oxidizing capacity of a deoxycholate-treated heart muscle preparation. It was concluded that cytochrome-*c* serves as an obligate mediator in the passage of electrons from succinate, DPNH or p-phenylenediamine to oxygen.

The current notion of the role of the cytochrome components in electron transport is shown in Reaction 7.



*Classification and nomenclature.*—Some of the common characteristics of the known cytochromes have been listed in Table II. The original references must be consulted for data such as pH, and temperature at which these constants were established.

In recent years there has been much discussion concerning the method to be used in the naming of cytochrome components. The word cytochrome itself would appear to have been a poor choice because it imparts no knowledge of either the structure or function of the enzyme. Egami *et al.* (138) have put forth a series of proposals for a revision in cytochrome terminology. These authors would prefer that the cytochromes be segregated on the basis of the heme type. However, since it will be some time before this information is available, a more effective procedure might be to adopt the term *electron transferase* (139) in place of *cytochrome*. Each component would still have to be labelled as to source since minor differences will always be encountered, for example, in isoelectric points. The function of the individual enzymes might be clarified by appending the intracellular  $E_0'$  (pH 7) since this information would automatically fix the position of the carrier in the

TABLE II  
SOME CHARACTERISTICS OF THE CYTOCHROME ENZYMES

Component	Source	Spectral Properties			$E_0'$ mv.	Remarks	Ref.
		$\alpha$	$\beta$ $\lambda, m\mu$	Soret			
a <sub>4</sub>	Acetobacter	612	—	—	—	—	(126)
a <sub>4</sub>	Widely distributed	600	—	448	—	The terminal cytochrome oxidase	(127)
a <sub>2</sub>	Bacteria	630	—	—	—	Autoxidizable	(128)
a <sub>1</sub>	Bacteria	590	540	427	—	Respiratory enzyme of certain bacteria	(128)
a	Widely distributed	603	—	452	+290	Closely associated with a <sub>4</sub>	(127)
c <sub>4</sub>	<i>Asotobacter</i>	555	526	420	320	High potential "c type"	(133)
c <sub>4</sub>	<i>Asotobacter</i>	551	522	416	300	High potential "c type"	(133)
c <sub>3</sub>	<i>Desulfovibrio</i>	553	525	419	-205	Low potential "c type"	(134)
c <sub>3</sub>	<i>Rhodospseudomonas</i>	550	521	415	340	Concerned in photosynthesis?	(135)
c <sub>1</sub>	Plants, heart	553	524	418	between band c	Transfers electrons to c	(127)
c	Widely distributed	550	521	415	260	Classical cytochrome; thoroughly characterized	(127)
b <sub>7</sub>	<i>Arum</i> spadix	560	529	—	- 30	Autoxidizable, cyanide "by-pass"	(127)
b <sub>6</sub>	Leaves	563	—	—	- 60	Possibly concerned in photosynthesis	(127)
b <sub>5</sub>	Liver and insects	556	526	423	25	Microsomal cytochrome	(129)
b <sub>4</sub>	Halotolerant bacteria	554	521	418	—	—	(130)
b <sub>3</sub>	Plants	560	529	—	+ 40	Autoxidizable, cyanide "by-pass"	(127)
b <sub>2</sub>	Yeast	557	528	423	—	Associated with lactic dehydrogenase	(131)
b <sub>1</sub>	Bacteria	560	530	426	+250	Involved in nitrate reduction	(128)
b	Widely distributed	563	530	432	- 40	May not be on direct electron transfer path	(132)
"c type"	Microorganisms		varies		varies	These components illustrate the need for an improved nomenclature.	(136)
dh	Young roots	571	—	424	20 to 50	Not a cytochrome; a peroxidase complex	(137)
f	Chloroplasts	555	526	422	365	Concerned in photosynthesis	(127)
h	<i>Helix</i> snail	556	526	422	—	Respiratory carrier?	(117)

electron transport chain. The cytochromes represent a class of enzymes for which the  $E_0'$  can be easily established in many cases by the use of oxidation-reduction buffers. Finally, as more information became available, the active center could be designated according to the type of metal and prosthetic group present in the molecule. According to this system the classical cytochrome-*c* would become *beef heart iron porphyrin electron transferase*<sup>+0.260</sup>.

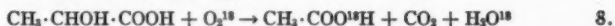
#### OXYGEN METABOLISM

In recent years evidence has accumulated to indicate that molecular

oxygen plays a more diverse role in tissue metabolism than has hitherto been suspected. Thus in addition to serving as the terminus needed for trapping electrons generated by the energy-yielding cellular processes, molecular oxygen may be transferred by a variety of enzymes to a variety of structures.

In reviews of the mechanism of oxygen metabolism, Mason (140, 141) defines three separate classes of enzymes concerned with this process. The first class, *oxygen transferases*, catalyze the transfer of an entire molecule of oxygen to substrate. The second class, *mixed function oxidases*, catalyze the transfer of one atom of oxygen to the substrate, the other atom undergoing reduction to water. The third class, *electron transferases*, catalyze the reduction of molecular oxygen to hydrogen peroxide or water. Specific examples of the three classes would be pyrocatechase, lactic oxidative decarboxylase (see below) and cytochrome oxidase, respectively. Recently Mason *et al.* (142) have found horseradish peroxidase to catalyze the hydroxylation of a number of aromatic cosubstrates in the presence of dihydroxy fumarate and oxygen. The enzyme was at least 1000 times as active as ferrous iron or hemin. A positively charged oxygen complex of horseradish peroxidase has been suggested as a likely intermediate. Mason *et al.* (143) report the hydroxylation of salicylic acid in this system with oxygen supplied as either  $O_2^{18}$  or  $H_2O^{18}$ . This experiment demonstrated unequivocally the capacity of the enzyme to transfer oxygen. The authors suggested oxyferroperoxidase as a possible intermediate.

In place of the terms *oxygen transferase* and *mixed-function oxidase*, Hayaishi (144) prefers the name *oxygenase*. The latter is alleged to be somewhat more succinct and consistent with the term *hydrogenase* and would be applied to any enzyme either adding both atoms of molecular oxygen to substrate or adding only one atom of oxygen while reducing the other to water. In the case of the crystalline lactic oxidative decarboxylase of *Mycobacterium phlei*, (145) the mechanism (Reaction 8) would be that of a mixed function oxidase (Mason terminology).



The most recent communication from Hayaishi's laboratory describes the incorporation of  $O_2^{18}$  into the cellular material of a growing culture of *Pseudomonas* (146). With aromatic substrates as carbon sources, from 4 to 9 per cent of the oxygen of cell constituents was derived from atmospheric oxygen.

In a particularly informative résumé of his work with oxygen consumption in the respiratory chain, Chance (147) has pointed out that no changes in the activity of this system will be encountered until the  $O_2$  concentration has fallen to about  $4 \mu M$  at  $25^\circ$ . A polarographic technique suitable for simultaneously measuring both respiration rate and oxygen concentration in suspensions of rat liver cells has been described by Longmuir (148). Balázs (149) has devised an electrometric respirometer for measurement of the amount of oxygen in a given air space with a sensitivity of 0.05 to 0.1 per cent.



A heat stable agent capable of substituting for oxygen in the reactivation of anabiotic yeast cells has been found in yeast cell juice (150). Hyperoxidation appears to exert a deleterious effect on glutamine synthetase in brain and liver, the enzyme from the latter source being particularly sensitive (151).

#### OXIDATIVE PHOSPHORYLATION

*Mechanisms.*—The subject of oxidative phosphorylation continues to be one of the most actively investigated in current biochemistry. One session of the International Symposium on Enzyme Chemistry, in Tokyo, October 1957, contained papers by Slater (152), Chance (153), Lehninger (154), and Boyer (155). In addition, attention is directed to the review by Martius (156) on the role of fat-soluble vitamins in oxidative phosphorylation.

Within the area of oxidative phosphorylation itself, the most intriguing question is still that of the site and mechanism of energy conservation. Some disagreement appears to exist between Chance and Lehninger concerning this phase of the reaction.

Chance (157) has emphasized the central role of ADP in oxidative phosphorylation and he has devised experimental methods to demonstrate the effect of the addition of this nucleoside diphosphate to guinea pig mitochondria. These experiments prove the direct participation of ADP as phosphate acceptor in oxidative phosphorylation and rule out the participation of adenylate kinase as the rate-limiting step.

The exchange of  $P^{32}$  with the terminal phosphate of ATP in the absence of net electron transport has been examined by Wadkins & Lehninger (158) and by Cooper & Lehninger (159). The exchange rate is maximum when the carriers are in the oxidized state and the authors (158) state that the evidence indicates "that the primary 'high energy' linkage generated during electron transport involves or is dependent upon the oxidized state of the respiratory carriers."

However, on the basis of the rate of interaction (see above) of ADP and uncoupling agents with the respiratory carriers, Chance & Hollinger (160) conclude that there are two intermediates between the adenylic acid system and the *reduced* form of the carriers.

Green *et al.* (161) emphasize that the almost exclusive use of liver mitochondria for studies of oxidative phosphorylation has long delayed recognition of the facts (a) that instability is not necessarily an invariant attribute of the phosphorylating system; (b) heart is a far superior source for a stable phosphorylating system; and (c) slaughter house material can be used successfully for preparation of actively phosphorylating mitochondria.

Vignais *et al.* (162) report phosphorylation quotients (P/O) of 1.4 to 3.4 for the oxidation of isocitrate in liver mitochondria. Studies on the rates of phosphorylation in tissues from rats three months and from two to three years old revealed (163) that in liver mitochondria the activity decreases 30 per cent with age but remains constant in the brain. No uncoupling with

age was observed in either tissue. However, the decreased activity in the brain supports the concept of aging as a decline in the metabolic activities of the cell. Bronk & Kielley (164) show that a number of divalent metal cations may replace  $Mg^{++}$  in oxidative phosphorylation by fragmented liver mitochondria. The various ions tested also activated the ATP- $P^{32}$  exchange reaction and the ATPase activity. Thus the activating ion may complex with ATP for phosphorylation or with ATP for the exchange or hydrolysis reactions.

A direct phosphorylation coupled to TPNH oxidation has been observed by Joshi *et al.* (165) in heart mitochondria supplemented with a soluble extract of heart (165). Pinchat (166) reports that natural polynucleotides or polymers of the type described previously by Grunberg-Manago *et al.* (167), i.e., from adenosine, uridine, or cytidine diphosphates, stimulate oxidative phosphorylation on DPNH.

Evidence has been found that the citric acid cycle oxidations of the phosphorylating electron particle which are induced by external DPN are not coupled to phosphorylation whereas the oxidation involving bound pyridine nucleotide is coupled (168).

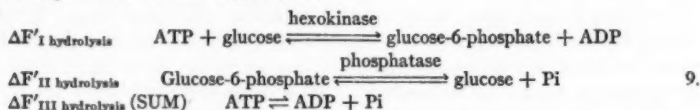
Several investigators have studied oxidative phosphorylation in plant preparations. Under appropriate conditions, P/O ratios greater than 3 were observed by Conn & Young (169) for the oxidation of  $\alpha$ -ketoglutarate to succinate in lupine mitochondria. Ratios approaching 2 were found for succinate. Thus it would appear that the lupine mitochondria phosphorylate with an efficiency comparable to that of animal mitochondria. Soybean mitochondria oxidize many Krebs cycle intermediates although fumarate and oxaloacetate are oxidized slowly (170). Hexokinase increased the P/O ratio for succinate, pyruvate and  $\alpha$ -ketoglutarate while sodium fluoride decreased oxygen uptake without affecting the P/O ration. In *Chlorella* the route of phosphate incorporation proceeds from inorganic phosphate to ATP to protein phosphate (171).

Recently, the effect of a number of new inhibitors and uncoupling agents for oxidative phosphorylation has been explored. Zetterström & Ernster (172) find bilirubin, but not biliverdin, to uncouple phosphorylation in mitochondria at a level of  $3 \times 10^{-4}M$ . The  $Ca^{++}$  inhibition of oxidation of DPN-linked substrates in mitochondria is reduced by substituting AMP for hexokinase-glucose as the phosphate acceptor (173, 174). Injection of 2,4-dinitrophenol or Janus Green B into living rats resulted in an increase in both mitochondrial and nucleolar ATPase (175). The uncoupling effect of phalloidine at the cytochrome step has been noted by Hess (176). Half maximum effect was found at  $9 \times 10^{-6}M$ . Maley (177) has been able to show that mitochondria from thyrotoxic rat liver oxidize external DPNH with greatly reduced P/O ratios.

**Phosphagens.**—Recent years have also seen the appearance of new phosphagens. The isolation of natural guanidophosphoamides such as glycocya-

mine phosphate, taurocyamine and lombricine (guanidoethylserylphosphate) has been reported (178). A survey of a number of species revealed that, contrary to popular opinion, there is no connection between the type of compound present and zoological classification. Specific phosphokinases for taurocyamine and phosphoglycocycamine occur in *Arenicola marina* and *Neris diversicolor*, respectively (179). Krishnan *et al.* (180) propose that soluble metaphosphate functions as a comparatively inert phosphagen, gradually accumulating and then disappearing. Insoluble metaphosphate on the other hand is thought to serve as an active phosphagen in conjunction with ribonucleic acid, in the synthesis of cellular material. Crayfish arginine phosphokinase has been highly purified by Morrison *et al.* (181) The pH optima in both directions, SH nature and other molecular and kinetic properties of the enzyme were established. It was also shown that the enzyme phosphorylates homoarginine and canavanine although a number of other acceptors, including AMP, were inactive.

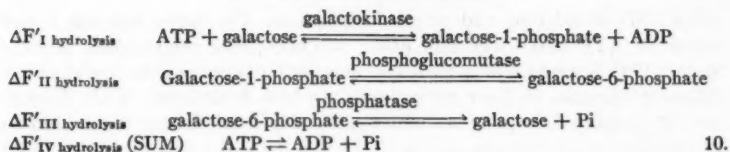
$\Delta F'_{\text{hydrolysis of ATP}}$ .—Two laboratories have carried out a more or less direct determination of the apparent energy of hydrolysis of ATP by means of the sequence shown in Reaction 9.



Since  $\Delta F'_{\text{II hydrolysis}}$  is known,  $\Delta F'_{\text{III hydrolysis}}$  can be estimated through measurement of the equilibrium constant of reaction I with hexokinase. Robbins & Boyer (182) employed a number of devices for determination of this equilibrium. These included the use of a lower pH and isotopic dilution for measurement of glucose- $\text{C}^{14}$ . Evaluation of the apparent equilibrium constant at physiological pH gave values of  $-7.6$  and  $-7.8$  kcal. mole $^{-1}$  for the dephosphorylation of ATP at zero and excess  $\text{Mg}^{++}$ , respectively. Vladimirov *et al.* (183) used  $\text{P}^{32}$  as an analytical aid in determining the equilibrium of the hexokinase reaction. Their results lead to a value of  $-5.6$  kcal. for the apparent free energy of hydrolysis of ATP at pH 7.25.

A somewhat more effective method for determination of the free energy of hydrolysis of ATP might be found in the following series of equations: (Reaction 10).

\* Students of biochemistry should not fail to read the recent review of energetics by Krebs & Kornberg (233). This paper, which may be regarded as a sequel to the now classical articles by Lipmann (234, 235), is well organized and is written in a simple and comprehensible style. The paper begins with a restatement of the central role of ATP in metabolism and then proceeds to trace the biosynthesis of this substance starting at the level of protein, fat and carbohydrate. The article reviews photosynthesis as well as the standard and alternate pathways of carbohydrate metabolism and concludes with a scheme for the evolution of energy transforming mechanisms.



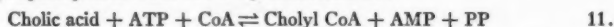
Since

$$\Delta F'_{\text{IV hydrolysis}} = \Delta F'_{\text{I hydrolysis}} + \Delta F'_{\text{II hydrolysis}} + \Delta F'_{\text{III hydrolysis}}$$

and since  $\Delta F'_{\text{II hydrolysis}}$  (phosphoglucomutase acts on galactose-1-phosphate) and  $\Delta F'_{\text{III hydrolysis}}$  are known, only  $\Delta F'_{\text{I hydrolysis}}$  need to be measured.

Unfortunately, many biochemists have failed to realize the importance of  $\text{H}^+$  in the apparent free energy of dephosphorylation of nucleoside polyphosphates, a fact clearly pointed out in 1951 by Alberty *et al.* (184). Recently, Carpenter (185) has suggested that the designation  $\Delta F^{\circ}_{\text{hydrolysis}}$  be reserved for the case where all solutes are in the standard state and that an "un-ionized convention" be adopted as a universal standard for describing the energetics of hydrolytic reactions.

The universal role of ATP as an activating agent is substantiated by the finding of new reactions involving "high energy" metabolites. According to Elliott (186) the cholic acid activating system of washed microsomes forms AMP and pyrophosphate from ATP by Reaction 11:



Bachhawat & Coon (187) have provided further information on the  $\text{CO}_2$  activating enzyme. It is assumed that the reactive intermediate which is formed at the expense of ATP and which can carboxylate  $\beta$ -hydroxyisovaleryl CoA is adenylylcarbonate.

*Metabolism of the adenosine polyphosphates.*—A very great number of papers deal with some aspect of the metabolism of the adenosine polyphosphates. Of particular interest is the reported crystallization of myokinase, i.e., the ATP-AMP transphosphorylase of muscle (188, 189). The molecular weight (21,000) and other physical constants for the enzyme have been reported. Smillie (190) concludes that the major pathway for the conversion of AMP to inosine in brain homogenates is via inosine monophosphate rather than adenosine. The principal enzymes involved in the breakdown of ATP in this tissue appear to be adenosine triphosphatase, myokinase, 5' adenylic deaminase, adenosine deaminase and 5' nucleotide phosphatase. Cooper & Lehninger (191) have studied the ATPase activity of a digitonin extract of rat liver mitochondria. The possibility is discussed that the ATPase represents a hydrolytic dysfunction of the enzyme system which reversibly transfers phosphate from a hypothetical high energy ester to ADP. The effect of inhibitors and metal ions on the 5' adenylic acid phosphatase of microbial and plant tissue has been studied by Bargoni & Luzzati (192) while Hurwitz *et al.* (193) have characterized an enzyme from *Azotobacter vinelandii* which

splits AMP to adenine and ribose-5-phosphate. The latter enzyme is activated by ATP and by certain other polyphosphate compounds. Myers & Slater (194) describe, under their characteristic pH optima, no less than four different enzymes in liver mitochondria which hydrolyzes ATP. Dinitrophenol stimulated the enzymes with optima at pH 6.3, 7.4 and 8.5 but did not affect the enzyme with pH optima of 9.4. Centrifugal separation of the ATP-ase and triphosphatase activities of rat liver homogenates has been achieved by Fodor & Lehrman (195). Two soluble tri-phosphatases were detected, one of which appears to be an SH enzyme. Cohn & Meek (196) have used  $H_2O^{18}$  to investigate the mechanism of ADP and ATP hydrolysis catalyzed by potato apyrase. Cleavage occurs between oxygen and terminal phosphate of each substrate. Lee *et al.* (197) found no adenylate kinase activity associated with the adenylpyrophosphatase of potato. The resynthesis of ATP from AMP and ADP has been demonstrated in a glucose-free suspension of human erythrocytes following the addition of inosine or adenosine (198).

## MISCELLANEOUS TOPICS

### METABOLIC CYCLES

*Glyoxylate cycle.*—A major achievement has been the elucidation of the mechanism whereby microbial cells can grow on 2-carbon compounds (199). The answer to this vexing problem was provided by the discovery, in several

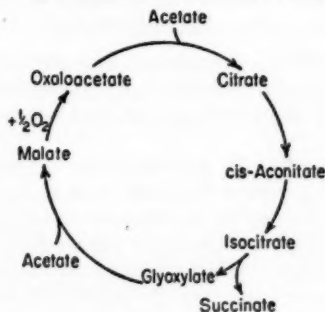
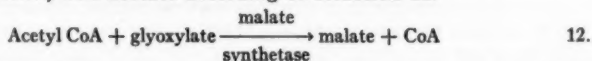


FIG. 6. The glyoxylate cycle according to Krebs & Kornberg (199).

laboratories, of two new enzyme systems. One of the new systems is the *isocitritase* which cleaves isocitrate to succinate and glyoxylate. The second enzyme, *malate synthetase* (200) forms malate from glyoxylate (from the isocitritase reaction) and acetate according to Reaction 12.



Krebs & Kornberg (199) suggest that these enzymes be incorporated into a new scheme, termed the *glyoxylate cycle* (Fig. 6). The effect of the cycle

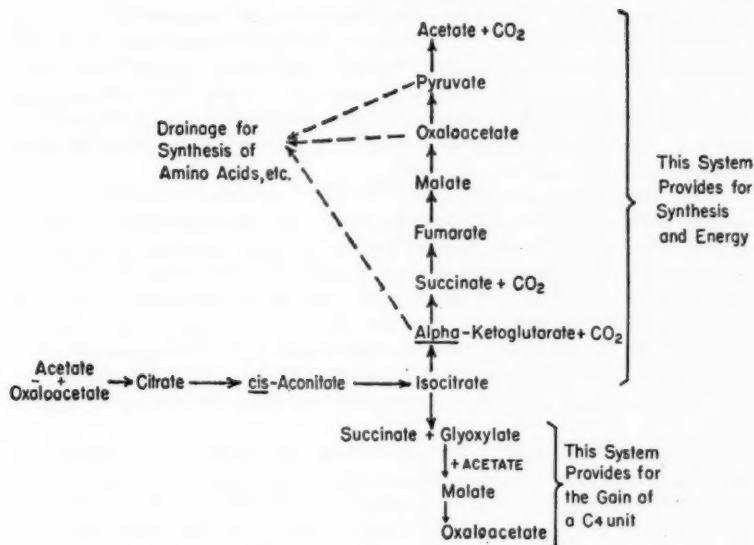


FIG. 7. Growth of bacteria on acetate, according to Wong & Ajl (200).

would be the formation of one mole of C<sub>4</sub> dicarboxylic acid from two molecules of acetate. Some of the other physiological implications of the glyoxylate cycle can be seen in Figure 7, taken from the paper by Wong and Ajl (200).

#### OXIDASES

The kinetics and mechanism of action of polyphenol oxidase from French prunes has been investigated by Ingraham (201). The Michaelis constant for oxygen is not equivalent to the equilibrium constant. The value of the latter constant was found to be approximately 1.5 per cent and it was concluded that the enzyme combines with oxygen before the hydrogen donor. In certain parts of tomato plants infected with *Fusarium lycopersici*, polyphenol oxidase activity after three weeks of infection was twice that of the controls whereas the ascorbic acid oxidase activity remained the same (202). A careful study of the action of tyrosinase on some purified proteins has indicated that all of the tyrosine residues of  $\alpha$ -lactalbumin are oxidized and none of the residues in  $\beta$ -lactoglobulin are attacked (208). Physicochemically homogeneous preparations of mushroom polyphenol oxidase have been obtained by Kertesz & Zito (204). The pale yellow enzyme had a sharp band at 283  $\mu$  ( $E_{1\text{cm}}^{1\text{ per cent}} = 27.55$ ) and a shoulder at 340  $\mu$ . The molecular weight by either copper determination or by sedimentation-diffusion analysis was 32 to 35  $\times 10^3$ . Evidence has been obtained that the copper always remains in the

cuprous state (204a). A direct relationship between formation of a quinone antibiotic and the presence of a laccase-type phenol oxidase has been shown in certain microbial species (205). The intermediates in the action of polyphenol oxidase on catechol have been examined by a paper chromatographic procedure (206). At low substrate concentrations only a purple red pigment could be detected but at higher substrate concentrations the three isomeric tetrahydroxydiphenyls were formed.

Hog kidney phenol oxidase was found to inactivate estradiol and stilbestrol (207). Using the agar-gel method of assay for tyrosinase, Cooper & Brown (208) have shown that the rate of melanin formation from 3,4-dihydroxyphenylalanine (dopa) is much greater than from tyrosine. Kazantseva & Kaplanskiĭ (209) report that the basic cause of disturbance in liver tyrosinase oxidation in protein deficiency is due to a reduction in the rate of formation of ketoglutaric acid in the citric acid cycle. Phenylpyruvic acid and phenylacetic were found to be weak inhibitors of mammalian tyrosinase whereas *p*-hydroxyphenylacetic acid had the most marked inhibitory effect (210).

Frieden & Maggiolo (211) suggest a scheme for the mechanism of action of ascorbic acid oxidase involving release of inhibitory copper from the enzyme and the production of a highly reactive intermediate in the oxidation of ascorbic acid. In experiments with etiolated pea internodes Eichenberger & Lhimann (212) showed by use of light reversal of CO inhibition that terminal respiration is mediated mainly through cytochrome oxidase rather than ascorbic acid oxidase.

Of a number of substrates related to glucose, only the 2-deoxy compound is actively attacked by glucose oxidase (213). The product was shown to be 2-deoxy-D-gluconic acid (214).

Certain amino acid hydrazides, such as L-leucylhydrazide act as effective inhibitors of diamine oxidase (215). Amine oxidase of plant source has been shown by Clarke & Mann (216) to convert tryptamine to 3-indole acetaldehyde. Faute *et al.* (217) conclude that the second basic group of a substrate for diamine oxidase is not essential for the formation of the enzyme-substrate complex but helps to form the complex through its nucleophilic character. The results of a study of the action of competitive inhibitors on D-amino acid oxidase is compatible with the hypothesis that in the formation of a ternary complex of protein, flavin coenzyme and substrate, the initial formation of oxidase is an obligatory step (218). The product of cystamine oxidation by pea seedlings was shown to be the cyclized form of the amino-aldehyde, cystaldimine (219).

A study of the substrate specificity of xanthine oxidase reveals that neither guanine or 7-methyl guanine are oxidized. However, both 2,6-diaminopurine and the oxidation product are inhibitors for the enzyme (220). Weanling rats maintained on diets marginally deficient in lysine appear to exhibit a deficiency in the capacity to synthesize the enzyme (221). The inhibitory ef-



fect of a large number of pteridines, purines and pyrimidines on liver and milk xanthine oxidase has been examined by Ricceri (222). A number of purine analogues have been tested by Fiegelson *et al.* (223) as inhibitors and substrates for xanthine oxidase. The enzyme was found to convert pyrazolo-adenine to pyrazoloisoguanine.

An ascorbic acid dependent DPNH oxidase of mammalian tissues has been studied by Kersten *et al.* (224).

#### HYDROPEROXIDASES

In a most interesting paper, Jensen (225) reports that a hemin-requiring mutant of *Micrococcus pyogenes* synthesizes apocatalase in hemin-free media. Resting cell suspensions of the organism couple hemin to the apoprotein. Coenzyme A plays an important role in the terminal synthesis and the possibility is discussed that hemin is transferred from an intermediate CoA-S-hemin compound to the apoenzyme. The intracellular catalase of *Micrococcus lysodeikticus* would seem to be insulated from pH changes in the external environment. The barrier appears to be the plasma membrane of the organism (226).

Japanese radish peroxidase has been crystallized by Morita & Kameda (227). The enzyme, which was found to be homogenous by physical methods, has a molecular weight of  $55 \times 10^3$ . Harbury (228) has measured the oxidation reduction potential of horseradish peroxidase as a function of pH. The simplest interpretation of the observed change in the mid-point potential with pH is that of an oxidation-linked proton equilibrium in the ferro enzyme with  $pK'$  of 7.38 and a corresponding equilibrium in the ferri enzyme with  $pK'$  of approximately 10.56. The same paper describes a useful titration assembly suitable for simultaneous collection of potentiometric and spectrophotometric data.

Polis & Shmukler (229) have presented the first detailed characterization of mitochrome, a hemoprotein released from aged mitochondria. The molecular weight by sedimentation and diffusion and by iron analysis is  $102$  to  $107 \times 10^3$ . The preparation is electrophoretically homogenous on either side of the isoelectric point, pH 5.1. There are two main absorption bands, at 410 and at 280  $m\mu$  respectively. Kinetic and tracer studies suggest that mitochrome serves to dephosphorylate ATP by discharging both phosphate and nucleotide from the surface of the mitochondrion.

The two main gaps in knowledge of heme biosyntheses are at either end of the process. The initial reaction between glycine and a 4-carbon intermediate is still obscure. However, working with cell suspensions of *T. vorax*, Lascelles (230) has found indication that pyridoxal and pantothenate may be directly involved at this stage. Shemin (231) suggests that it is likely that both glycine and succinate are activated by compound formation with co-enzymes. A specific inhibition of  $\delta$ -aminolevulinic acid syntheses could be overcome with pyridoxalphosphate.

The enzyme which transforms porphobilinogen to uroporphyrin III has been purified by Lockwood & Rimington (232) and named *porphobilinogenase*. Of considerable interest is the observation that gentle heat treatment of the enzyme results in the formation of uroporphyrin I. Hence it would appear that heating the enzyme has duplicated the damage found in the well-known physiological error in porphyrin metabolism.

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## PROTEOLYTIC ENZYMES<sup>1</sup>

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### INTRODUCTION

The application of modern methods of protein chemistry and enzymology to the study of the proteolytic enzymes is beginning to provide an interpretation of the catalytic function of some of these enzymes in terms of molecular structure. Accordingly, in the present review, primary emphasis has been placed on the characterization of proteolytic enzymes by physical, chemical and enzymatic means, and on a discussion of the active center and mode of action of trypsin and chymotrypsin. Certain subjects pertinent to the field of proteolytic enzymes have been deliberately omitted and others have been dealt with only briefly. Thus, enzymes involved in blood coagulation have not been considered, and the action of proteolytic enzymes on the primary structure of proteins has been considered herein only in brief. This seems to be justified, as in last year's review of *The Proteolytic Enzymes*, Jandorf & Michel (1) have discussed this subject in detail, and Behrens & Bromer (2), in the current volume, have considered the application of proteolytic enzymes to the determination of the structure of protein hormones. The problem of the activation of zymogens, though in the foreground of current research on proteolytic enzymes, has been the subject of recent detailed reviews (3, 4, 5) and, hence, only the most recent work will be considered herein. Other major reviews have appeared during the last year on the subject of proteolytic enzymes, including a Harvey Lecture by Fruton (6), a penetrating analysis of the problem of protein structure in relation to function and biosynthesis by Anfinsen & Redfield (7), and a monograph on *Proteolytic Enzymes and Their Clinical Applications* (8).

With a few exceptions, the survey covered by this review includes the literature available to the authors up to November 1, 1957. Abstracts of papers presented at meetings have been included only if they appeared to be of particular relevance to the topic under consideration. Nevertheless, in order to keep the present review within the allotted space, it has been necessary to observe discretion in the selection of papers being reviewed; in addition, errors of omission may have occurred, and hence no claim for complete coverage of the literature can be made.

### NEW ENZYMES AND NATURALLY OCCURRING INHIBITORS

This section has been constructed with a fairly wide interpretation of the adjective "new," and inclusion of an enzyme (or inhibitor) which has been

<sup>1</sup> The following abbreviations have been used in this review: Amino acid residues are denoted by the first three letters of their names. The following additional abbreviations have been used: FDNB for fluorodinitrobenzene; DFP for diisopropylphosphorofluoridate; DNP for dinitrophenyl; TCA for trichloroacetic acid.

described previously should not be interpreted as casting any doubt upon the priority of a previous description; rather, that what before has been perhaps a somewhat ill-defined activity has now reached the status of a more well-defined protein fraction, the enzymic activity of which has been clearly established.

#### EXOPEPTIDASES

The above comments apply particularly to the exopeptidase, cysteinyl-glycinase. This activity, together with some other peptidase activities, has been previously described as being associated with ribonucleotide fractions from hog kidney entirely free of protein, thus leading to the far-reaching claim that these polynucleotides possessed enzymic activity and were, therefore, the first nonprotein enzymes (9, 10, 11). In an important paper, Semenza (12) has carried out a rigorous chromatographic purification of such a preparation of cysteinyl-glycinase and has shown most elegantly that a protein enzyme of high turnover toward its substrate ( $C_1 = 2000$ ) may be isolated in a homogeneous state. This protein is orcinol-negative and contains no phosphate; it has a typical protein spectrum, is nondialyzable, and is rapidly inactivated by shaking with chloroform (in contrast to the impure preparations which are stable to this treatment). The activity is lost reversibly in 6*M* urea and is also lost irreversibly during proteolytic attack by chymotrypsin and trypsin in the presence of urea, although it is stable to these enzymes in the absence of urea. Ribonuclease is without effect under any conditions. It seems clear that the original preparations consisted of small amounts of very active enzyme protein adsorbed onto ribonucleic acid and thus considerably stabilized toward denaturing conditions. Similar claims of Binkley (11) concerning the nonprotein nature of leucine aminopeptidase have been refuted by Patterson (13), who purified the enzyme extensively from ascites tumor by digestion of the extracts with proteolytic enzymes and fractional precipitation. It is true that in addition to TCA- and bromsulfalein-precipitable protein, the preparation was then found to contain glucosamine and nucleoside mono- and diphosphates with guanine as the predominant base, in a way similar to the aminopeptidases prepared by Binkley. Nevertheless, in addition to a greater size of the polypeptide component than previously thought, the most purified ascites fractions exhibited leucine aminopeptidase activity in direct proportion to protein concentration, and no quantitative relationship of activity to other substances present could be detected.

An  $\epsilon$ -lysine acylase has been described by Paik *et al.* (14, 15) as being present in rat kidney, extracts of which were able to hydrolyze  $\epsilon$ -N-acetyl-L-lysine. The enzyme was concentrated approximately one hundredfold and was found most active in phosphate buffers at pH 7.0 to 7.2, there being no evidence of metal activation. It was more active on  $\epsilon$ -N-chloroacetyl-L-lysine than on  $\epsilon$ -N-acetyl-L-lysine and was inactive toward  $\epsilon$ -N-acetyl-D-lysine,  $\epsilon$ -carbobenoxyl-L-lysine, biocytin, and  $\delta$ -chloroacetyl-L-ornithine. No ex-

perimental data were presented on its action upon  $\epsilon$ -acetylated lysine residues in peptides or proteins where a positive result would have important implications in selectively freeing lysine residues in a protein modified by acetylation.

Mandl *et al.* (16) have described some metal-activated peptidases in the culture filtrates of *Clostridium histolyticum* which act upon N-terminal leucyl, glycyl, alanyl, prolyl, and phenylalanyl peptides. Upon ammonium sulfate precipitation the leucyl activity was enriched at the expense of the glycyl one. In contrast to Smith's leucine aminopeptidase (17), these leucyl peptidases did not hydrolyze leucinamide and were not activated by added  $Mg^{++}$  or  $Mn^{++}$ ; treatment with ethylenediaminetetracetic acid caused complete inhibition of all peptidase activity which could, however, be restored nonspecifically by all di- and trivalent metals tested.

A tripeptidase from human erythrocytes has been purified by Tsuboi *et al.* (18) in excess of one thousandfold, their best preparations having turnover numbers of 3200 and 18,000 moles of Gly.Gly.Gly and Ala.Gly.Gly per min. per 100,000 gm. protein. The specificity of the enzyme appears to be for tripeptides, there being no action on Gly.Gly; no metal activation was reported, but divalent metals ( $Co^{++}$ ,  $Mg^{++}$ ,  $Ba^{++}$ , and  $Ca^{++}$ ) had a protective action during the preparation. Dialysis against ethylenediaminetetracetic acid had little effect on activity, but there was considerable inhibition by *p*-chloromercuribenzoate, indicating involvement of  $-SH$  groups.

Erlanger (19) has isolated some ornithine peptidases from *Bacillus brevis*. These enzymes behaved as aminopeptidases, were activated by  $Mn^{++}$  or  $Co^{++}$ , and were specific for L-ornithine peptides. They were inhibited by phosphate, pyrophosphate, iodoacetate, and *p*-chloromercuribenzoate.

#### ENDOPEPTIDASES

**Pancreatic.**—Despite exhaustive work in the past several decades, the pancreas, the repository of so many endopeptidase activities, still yields new proteolytic activities, but we must be increasingly skeptical of their real existence until their nonidentity with known activities (or mixtures of activities) is firmly established.

Four new activities have been forthcoming in the past year, the first described by Weil *et al.* (20, 21) and having action upon protamines; the second, still undefined but being concerned in elastolysis; the third, an insulinase described by Lewis & Thiele (22); and, finally, Grant *et al.* have described an insoluble proteinase from hog pancreas which they have called "Pankrin" (23).

Weil *et al.* (21) have obtained a preparation from hog pancreas by making a glycerol extract of the acetone powder, adsorbing twice on alumina- $C_7$  and inhibiting with DFP. This preparation is known as protaminase and can remove C-terminal arginine from salmine and from the tryptic breakdown products of other proteins. It has no action on proteins such as  $\alpha$ -lactalbumin,

$\beta$ -lactoglobulin, or gelatin, which are devoid of C-terminal arginine or lysine, but prior hydrolysis with trypsin allows protaminase to liberate arginine or lysine. Their preparation was strongly activated by trypsin, and, following DFP inhibition, showed increased ability to hydrolyze salmine, casein, etc. Upon heat treatment at 100°C. and pH 2.3 for 60 min, there was a residual activity but the ability to liberate free arginine from salmine was lost. Thus, two separate activities could be distinguished, a heat-labile exopeptidase (or carboxypeptidase) capable of removing C-terminal arginine or lysine residues and which was present in the active state in the crude extract, and a heat-stable endopeptidase present only after tryptic activation of the crude extract. Folk (24) has previously described an enzyme which he called basic carboxypeptidase (or carboxypeptidase B) which was obtained in the zymogen form from fresh pancreas or an extract of acetone powder (beef) and was activatable by trypsin. Since Weil *et al.* state that their crude preparations were devoid of tryptic activity, it may be that the enzyme which was in the active state in their preparations is different from that described by Folk.

As for the trypsin-activatable activity described by Weil, it may well have a dual nature, one component being identical with Folk's carboxypeptidase B and the second, a heat-stable component, being an endopeptidase with a specificity similar to that of trypsin. In fact, this heat-stable endopeptidase possesses several features in common with trypsin: heat stability (or ready reversal of heat denaturation), specificity, and lack of appreciable further action on salmine which had been exhaustively hydrolyzed by trypsin. Two differences are also apparent: lack of DFP inhibition and lack of action upon synthetic ester substrates of trypsin. However, it is the unhappy experience of workers with trypsin (and indeed other proteolytic enzymes) that DFP inhibition tends to be slow and incomplete, and a level of trypsin which is undetectable by the esterase assay may have a rapid action upon protein substrates. A final conclusion as to the nature of this endopeptidase must, therefore, await further physicochemical characterization.

The field of elastolysis remains complex despite the isolation by Lewis *et al.* (25) of a crystalline "elastase" from pork pancreas powder. Electrophoretic and ultracentrifuge data were cited to support the homogeneity of this preparation, but a later note by Lewis & Thiele (22) showed that upon subjecting crystalline elastase to chromatography on diethylaminoethyl-cellulose, five components were apparent. The elastase remained in a cationic fraction which was not held up at pH 8.8. In the former paper is given a detailed list of the molecular parameters of crystalline "elastase" and a most striking resemblance to  $\alpha$ -chymotrypsinogen is evident: crystal form, isoelectric point, sedimentation and diffusion constants, molecular weight, all are identical. Two differences are, however, apparent;  $E_{280}^{1\%}$  is much lower (11.0 versus 20.0) and the preparation is labile at low pH's where  $\alpha$ -chymotrypsinogen is stable.

Hall (26) has indicated that two separate enzymes in hog pancreas act

synergistically in elastolysis, and suggests that the explanation may lie in the structure of elastin. One enzyme is concerned in hydrolysis of the mucopolysaccharide envelope which surrounds a central protein fibril, and the other in the proteolysis of the central fibril. He has been able to separate these two activities by paper electrophoresis, and in contrast to Lewis *et al.* (25), finds them to have isoelectric points close to neutrality. Grant & Robbins (27) have also obtained an "elastase" preparation from pork pancreas (Pankreatin) by adsorption of the enzyme onto, and elution from, elastin. They found that their purest preparations have strong activity both upon elastin and acetyl tyrosine ethyl ester, thus indicating either a strong contamination or a common specificity with chymotrypsin. It seems clear, therefore, that at the present time no single well-defined protein fraction can yet be identified with the elastolytic activity of pancreas.

*Pancreatic insulinase.*—Lewis & Thiele (22) have separated an anionic component from their elastase preparations by chromatography on diethylaminoethyl-cellulose which has high activity in solubilizing the  $I^{131}$  into the TCA supernatant solution from  $I^{131}$ -labeled insulin; because of this they have called it pancreatic insulinase. The present authors would like to echo the comments of last year's reviewers (1) in urging caution in the interpretation of this assay for insulinase. A serious pitfall is apparent since a mixture of an endopeptidase, having the specificity of chymotrypsin, with some pancreatic carboxypeptidase (Anson) would be very effective in releasing tyrosine or tryptophan-bound  $I^{131}$  from insulin. The former enzyme would split the peptide chain on the carboxyl side of tyrosine or tryptophan, leaving these aromatic residues in C-terminal positions, and carboxypeptidase would remove the corresponding free amino acids very rapidly. Thus, such a mixture of enzymes would have the properties of an insulinase as defined by this test but would be in no way specific for insulin.

*Pankrin.*—Grant & Robbins (23) have described the partial purification of a water-insoluble proteinase from hog pancreas (pancreatin) by adsorption of the enzyme from a water extract onto IRC 50 (XE 97) and by elution on increase of the ionic strength. They have attempted to differentiate the enzyme from other pancreatic proteases by showing that it possesses a higher ratio of activity toward proteins than toward the synthetic substrates for either trypsin or chymotrypsin. However, esterase activity toward both acetyl tyrosine ethyl ester and toluylsulfonil arginine methyl ester was present in the preparation and the protease activity was not completely inhibited either by trypsin inhibitors (soy bean or pancreatic) or by  $\beta$ -phenylpropionate. Since it is known that the effect of mixtures of proteases is far greater than merely the sum of their activities, it is open to question whether, in fact, a mixture of trypsin and chymotrypsin, possibly together with some other activities, adsorbed onto an insoluble protein (or mucoprotein) would not have similar properties to "Pankrin."

*Liver insulinase.*—Mirsky & Perisutti (28) have studied the "insulinase"

activity of liver by the same assay method as above, but have also used a number of other  $I^{131}$ -labeled proteins: casein, ribonuclease, ACTH, growth hormone and glucagon, and have found  $I^{131}$  to be released from all with rates ranging from 27 per cent for casein to 316 per cent for glucagon (insulin, 100 per cent). It is felt by the present reviewers that such a comparison between different proteins has very limited validity because it ignores the differing contents of residues which would be labeled with  $I^{131}$ , and more important, the strong effect of secondary and tertiary structure upon the rate of proteolysis of different native proteins. For example, glucagon and ACTH are probably random coils and thus very susceptible to proteolysis, whereas other proteins are probably highly folded in the native state and much more resistant to proteolysis. On the basis of a differential effect of aging of liver extracts or of the presence of citrate ions upon the rate of  $I^{131}$  solubilization, these authors have suggested that a specific proteolytic activity is responsible for the degradation of insulin.

*Skin endopeptidase.*—Martin & Axelrod (29) have extracted an endopeptidase from acetone powders of rat skin by using buffers of high ionic strength (1.6). This enzyme has the curious property of being active only at these high ionic strengths and being inactivated (reversibly) at lower ionic strengths. To explain this behavior the authors suggest that an enzyme-inhibitor complex is present in the extract which is dissociated at high ionic strengths but reassociates upon dilution. Other proteinases in skin which can attack acetyl tyrosine ethyl ester and tyrosine ethyl ester have also been described by Golubow (30).

*Liver endopeptidase.*—The intracellular distribution of proteases and peptidases in liver cells has been studied by Rademaker & Soons (31) using a differential centrifugation technique. They were able to show that  $Co^{++}$ -activated glycylglycine dipeptidase, a non metal-activated tripeptidase, and an endopeptidase acting on hemoglobin were present in the supernatant fraction. Cathepsin A, as measured by the splitting of carbobenzoxy-L-glutamyl-L-tyrosine, and carboxypeptidase, as measured by the liberation of phenylalanine from carbobenzoxy-glycine-L-phenylalanine were present in the mitochondria, the latter activity being mainly in the heavy mitochondria. Cathepsin B (benzoyl-L-argininamide as substrate) was distributed throughout the fractions. Finkenstaedt (32) has shown that liver mitochondria contain cathepsins B and C, while an inhibitor from cathepsin B is present in the supernatant fraction.

*Insect endopeptidase.*—Evans (33) has described a proteinase prepared from an aqueous extract of the dried gut of the blowfly, *Calliphora erythrocephala*, which is active on azocasein.

#### ENDOPEPTIDASES FROM MICROORGANISMS, FUNGI, AND PLANTS

Yoshida & Nagasara (34) have purified and crystallized an interesting proteinase from *Aspergillus saitoi* which appears to resemble pepsin in having



an optimum pH for casein digestion of 2.5 to 3.0. The crystalline material has approximately one-half the activity of crystalline pepsin toward casein.

Okunuki *et al.* (35) have described the specificity of a crystalline bacterial proteinase, while Reinartz & Hug (36) have isolated, by adsorption onto alumina, a proteolytic enzyme from yeast autolyzed with acid or chloroform, and have found it to be stable to oxidation by  $\text{H}_2\text{O}_2$  or to heavy metals such as  $\text{Hg}^{++}$  or  $\text{Cu}^{++}$ , indicating its non-sulfhydryl nature. Colobert (37) has prepared a proteinase from *Coccus P* (sp. *Sarcina flava*) by ammonium sulfate fractionation followed by ethanol- $\text{Ca}^{++}$  precipitation; the preparation was homogeneous in the ultracentrifuge, by electrophoresis and by solubility. O'Brien & Campbell (38) have studied a proteinase which is produced by the thermophilic bacterium *Bacillus stearothermophilus* (Strain 1503) at 55°C. Partial purification was achieved and the properties of the enzyme were as follows: the optimum pH was 6.9 to 7.2 and  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  was required for activity on casein. Synthetic substrates that were attacked were L-Leu.Gly. Gly, triglycine, tetraglycine, and glutathione. Sulfhydryl inhibitors caused inhibition which could be reversed by glutathione. The enzyme was stable at 55°C. but inactivated at 65°C.

An intracellular proteinase, bound to insoluble cell material, has been described by Prescott & Willms (39) as being present in *Micrococcus freudenreichii*. The active enzyme was released into solution following autolysis of a suspension of the washed cell debris.

Prado *et al.* (40) have described a cysteine-activated protease from the culture medium of *Cl. histolyticum* which had a specificity similar to that of trypsin, was able to coagulate milk, but was not inhibited by DFP. When fraction IV-4 of bovine plasma was treated with this enzyme, a pharmacologically active material resembling bradykinin was released.

Koch & Dedic (41) have carried out a survey of the proteolytic activities of a number of molds, e.g., *Penicillium* sp.

*Plant proteinases.*—A proteolytic enzyme has been demonstrated in potato juice by Niemann (42, 43) which acts upon gelatin, hemoglobin, casein, etc. The enzyme could be concentrated by ammonium sulfate precipitation, and in its dependence on pH and activation resembled papain.

#### NATURALLY OCCURRING INHIBITORS

Laskowski *et al.* (44) have reported that swine colostrum contains a trypsin inhibitor in much higher concentrations than human or bovine, colostrum, the concentration being maximal one day after birth and falling to zero by the fifth day. The inhibitor was partially purified by chromatography on diethylaminoethyl- and carboxymethyl-cellulose and then reacted with trypsin. The inhibitor-trypsin complex had an isoelectric point of 8.3 and chromatographed as a symmetrical peak on carboxymethyl-cellulose. The complex was dissociated by 2.5 per cent TCA, with precipitation of trypsin; the inhibitor remained soluble and could be crystallized from the supernatant solution.

The gut roundworm, *Ascaris lumbricoides*, by virtue of its existence in a highly proteolytic medium is a logical source of endopeptidase inhibitors, and Green (45) has shown that pepsin, trypsin, and chymotrypsin inhibitors are present. He has partially purified the chymotrypsin inhibitor and has found that it inhibits chymotrypsin stoichiometrically with a  $K_{dis.}$  of  $10^{-12}$  *M* and competes with the synthetic substrate, acetyl tyrosine ethyl ester for the enzyme. The reaction is second order and about forty times faster than in the pancreatic trypsin inhibitor-trypsin case.

Hilliard & West (46) have found a pepsin inhibitor in pituitary extracts, there being some correlation between thyrotropic potency and inhibitor concentration, but the activity has not been further purified.

## CHARACTERIZATION OF PREVIOUSLY KNOWN PROTEOLYTIC ENZYMES

### PHYSICAL AND CHEMICAL CHARACTERIZATION

The proteolytic enzymes of the extracellular secretions have continued to be the subject of extensive studies, and among these, pepsin and chymotrypsin have been particularly favored.

*Pepsinogen and pepsin.*—Dieu (47) has investigated the change in hydrodynamic properties of pepsin with pH and has shown that three regions can be discerned: below pH 5 to 5.5, the molecules are active; above pH 7.9 they are inactive and this change is accompanied by a lowering of  $S_{20}$  and  $D_{20}$ ; in both cases the sedimentation is nevertheless characteristic of a homogeneous substance. In the intermediate region, on the contrary, complication of the sedimentation diagram and electrophoretic pattern occurs, possibly due to the action of remaining active molecules on the denatured ones. Orekhovich *et al.* (48) have also observed a multiplicity of components of pepsinogen at low ionic strength, not only at pH 5.9 and 7.5 but also at pH 8.7, all components yielding, on activation, pepsins of comparable activity. With due consideration of the above, Orekhovich & Shpikiter (49) determined the sedimentation and diffusion constants, specific volume, and axial ratio of pepsinogen and pepsin, obtaining molecular weights of 42,240 and 39,930 for the zymogen and the enzyme, respectively, which is in satisfactory agreement with previous results.

In continuation of previous chemical studies on pepsin, Perlmann (50) has investigated the effect of urea on this enzyme and has shown that a marked loss of enzymatic activity occurs if pepsin is maintained in urea solutions at temperatures above 20°, whereas earlier work by Steinhardt (51) had led to the conclusion that at lower temperatures (3°) the enzyme is resistant to urea inactivation. The loss of activity, moreover, was accompanied by changes in the ultraviolet absorption of the protein, in its electrophoretic properties, and in its solubility in TCA, indicating that a change in the

tertiary structure, probably involving carboxyl and tyrosine residues, had occurred. These conclusions are also supported by kinetic studies by Edelhoch (52) on the effect of urea analogues and metals on the variation of the rate of pepsin denaturation at room temperature as a function of pH.

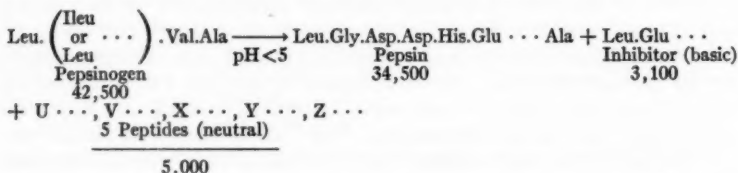
An elucidation of the chemical events in the conversion of pepsinogen to pepsin has been undertaken by Van Vunakis & Herriott. The inhibitor was isolated from activation mixtures using ion exchange resins, and its amino acid composition was determined (53). The FDNB method of Sanger was used for end-group determination, and the finding of only one N-terminal amino acid residue, identified as leucine, suggests that the molecule consists of a single peptide chain. The recovery of DNP-leucine would indicate a value of 3100 for the molecular weight of the inhibitor, while the value 3242 was deduced from amino acid analysis, in agreement with previous determinations. One feature of the amino acid analysis which is worthy of note is a high lysine content, compared with that of pepsin which is unusually low in all basic amino acids.

Similar methods were also applied to pepsinogen and pepsin (54). The amino acid identified as the N-terminal residue of pepsinogen was leucine, while isoleucine was found at the corresponding position of pepsin. The finding of different, single, N-terminal residues in both preparations suggests that both molecules are composed of single peptide chains and that pepsin does not occupy the N-terminal position in the precursor, pepsinogen. This was also shown to be true for the inhibitor from further studies of the amino acid sequence near the N-terminal portion of this polypeptide as compared to pepsinogen. The amino acid composition of pepsinogen, when compared to that of pepsin, again revealed a higher lysine content.

The purification of pepsin and the determination of its N-terminal residues have also been examined by Heirwegh & Edman (55). Using ion exchange resins, considerable progress seems to have been made in the difficult task of preparing pepsin solutions free of small peptides, these latter presumably being formed by the autolysis of the enzyme. The preparation thus obtained was treated by an improved modification of the Edman procedure, the results showing unequivocally that isoleucine is the sole N-terminal amino acid residue of swine pepsin, in agreement with the results by Van Vunakis & Herriott (above) and in disagreement with those of Williamson & Passmann (56), who found leucine as the N-terminal residue. Orekhovich *et al.* (48) have also confirmed the finding of N-terminal leucine and isoleucine in pepsinogen and pepsin, respectively, while treatment with carboxypeptidase led to the liberation of alanine, as previously described (57), followed by valine as the C-terminal amino acid of pepsinogen.

Finally, the immunochemical properties of different pepsin and pepsinogen preparations have been studied by Lobachevskaya (58), who concludes that only limited structural changes occur during activation. The data pres-

ently available on the chemical structures in the pepsinogen-pepsin system from swine can thus be summarized as follows:



The different fractions of pepsin preparations were extensively investigated by Veremeenko (59), with the conclusion that pepsin, as obtained from the autolysate of swine gastric mucosa, constitutes a mixture of proteins possessing peptic activity; but individual components, however, possess different physicochemical properties, such as different solubilities and different specific activities.

The isolation and purification of whale pepsin has been described by Saito & Ishihara (60).

*Chymotrypsinogen and chymotrypsin.*—The physicochemical properties of chymotrypsin have been investigated in considerable detail by Egan *et al.* (61, 62). By using countercurrent electrophoresis (63) the authors were able to show that commercial preparations of chymotrypsin as well as those that had been repeatedly crystallized were electrophoretically heterogeneous in the pH range 2.4 to 8.6, and that the individual peaks obtained on prolonged electrophoresis differed in their specific chymotryptic (esterase) activity. When the changes in the electrophoretic mobility of the peak associated with maximum enzymatic activity were correlated with the pH-mobility curves obtained for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chymotrypsin and for mixtures of these components, it was concluded that the heterogeneity of  $\alpha$ -chymotrypsin was due to the presence of variable amounts of the  $\beta$ - and  $\gamma$ -species, the  $\alpha$ -component amounting, in ideal conditions, to 91 per cent of the total protein content. The ultracentrifugal behavior of  $\alpha$ -chymotrypsin, which is known to dimerize in solution (64), was also examined and the relationship between pH and rate of sedimentation before and after photooxidation was studied. Complete loss of dimerization between pH 3.6 and 2.3 points to the involvement of a carboxylate group—probably contributed by aspartic acid—in the formation of the dimer, while loss of ability to dimerize after photooxidation, when one of the two histidines of the molecule is destroyed, is consistent with the hypothesis that a charged imidazole group also plays an essential role in this process.

As a complement to these data, Tinoco (65) has defined a set of conditions wherein enzymatically active  $\alpha$ -chymotrypsin was monomeric. It seems clear that there are strong attractive forces between  $\alpha$ -chymotrypsin molecules in the pH range where they have maximum catalytic activity, leading to

appreciable polymerization in solutions normally used for kinetic experiments. However, use of solutions with an ionic strength of 0.1 or higher will keep  $\alpha$ -chymotrypsin nearly monomeric, thus simplifying the interpretation of kinetic experiments.

The molecular weight of crystalline  $\alpha$ -chymotrypsinogen has also been determined with great care from amino acid analysis, light scattering, and sedimentation-diffusion measurements by Wilcox *et al.* (66). The values obtained by the use of these methods are all in excellent agreement and, together with the recently reported x-ray estimate (67), converge toward 25,000 as the most probable value. At the same time, evidence for large alterations in the reactivity of prototropic groups in  $\alpha$ -chymotrypsinogen has recently been presented by Wilcox (68). Chemical analysis, bearing especially on the determination of the number of glutamic and aspartic acid residues, free and in the form of amides, and potentiometric titration of  $\alpha$ -chymotrypsinogen, guanidinated and acetylated chymotrypsinogen, showed that the ionization of six prototropic groups has been altered in the native protein and is observable only below pH 5. Spectrophotometric data on the ionization of tyrosine similarly indicate that two of the four tyrosine residues have an altered reactivity in the native protein.

The study of the chemical structure of chymotrypsinogen has also been continued. Not only has the chemical relationship between the different chymotrypsinogens and chymotrypsins been elucidated (5, 69), as is discussed elsewhere in this chapter, but systematic work has also been started on the overall structure of the chymotrypsin molecule. Wilcox *et al.* (70) have made an extensive study of the amino acid composition of  $\alpha$ -chymotrypsinogen, using mainly the chromatographic methods of Moore & Stein. Included in this work was a determination of cysteic acid in performic acid oxidized chymotrypsinogen, and a determination of amide nitrogen as indicated by the release of ammonia during acidic or basic hydrolysis. A modification of the method for the determination of amide groups, based on previous work by Chibnall & Rees (71, 72) and Grassmann *et al.* (73, 74), was also described. The analytical results so obtained not only provide data on the number of glutamine and asparagine residues in chymotrypsinogen but also corroborate the total amide-NH<sub>3</sub> analyses based on the release of ammonia by acid or base.

Meedom (75) has continued her previous studies (76) on the three fractions obtained after performic acid oxidation of  $\alpha$ -chymotrypsin. These appear to be open peptide chains: fraction A, a peptide of 13 amino acids, having N-terminal cysteic acid and C-terminal leucine; fraction B, a long peptide chain, with N-terminal leucine or isoleucine and C-terminal tyrosine; and fraction C, now stated to be a peptide of about 50 amino acids, having N-terminal alanine and C-terminal tyrosine. Nevertheless, in view of the difficulties inherent in the preparation of homogeneous chymotrypsinogen and chymotrypsin, which undergo autolysis so readily (69), some of these

results, especially those concerning the C-terminal portions of the molecule and its constituent peptides, probably need further confirmation. Similar considerations apply to the results of a study of the reduction of the disulfide bonds in  $\alpha$ -chymotrypsinogen by Egan & Gross (77). Reduction of the —S—S— bonds of the protein was accomplished with  $K_2SO_3$  in the presence of *p*-chloromercuribenzoate; the process was found to be associated with significant structural breakdown of the molecule, probably into five fragments of an average molecular weight of approximately 5000, although no additional  $\alpha$ -amino groups, reactive towards fluorodinitrobenzene, were found. These findings, if confirmed, would be of the utmost importance, since it has always been believed that  $\alpha$ -chymotrypsinogen was made up of one single, possibly semi-cyclic, polypeptide chain. A recent communication by Gabelotau *et al.* (78) also deals with the nature of the C-terminal residues of  $\alpha$ -chymotrypsinogen. In contradistinction to Meedom's above-mentioned results, leucine, as part of the sequence (Ala.Val)-Leu, was found to be liberated by carboxypeptidase from urea-denatured chymotrypsinogen and from the C chain of oxidized  $\alpha$ -chymotrypsin. This discrepancy again points toward the difficulties associated with this problem, which is thus still awaiting a more definitive and reproducible solution.

The preparation and characterization of several chemical derivatives of  $\alpha$ -chymotrypsinogen have been described by Chervenka & Wilcox (79, 80). Treatment with carbon disulfide gave a protein which was predominantly the monosubstituted product in which the  $\alpha$ -amino group was replaced by a dithiocarbamate group. When the derivative, which could be shown to be homogeneous and monomeric at pH 8.7, was exposed to a solution at pH 3, the dithiocarbamate decomposed to carbon disulfide and a protein similar to, if not identical with, native  $\alpha$ -chymotrypsinogen could be recovered and recrystallized in 65 per cent yield. Both the derivative and "reversed" protein were converted by trypsin into active chymotrypsin. On the other hand, by treatment with O-methylisourea, a specific reaction occurred by which the 13 lysine residues were converted to 13 homoarginine residues, while the  $\alpha$ -amino group of the N-terminal half-cystine residue did not react to an appreciable extent. The guanidinated protein was shown to be homogeneous and monomeric at pH 3.0, but there was evidence of molecular association at pH 8.7. Here again the modified protein could be converted by trypsin into an enzyme having a specific esterase activity equal to that of  $\alpha$ -chymotrypsin.

Studies on the terminal amino acid residues in chymotrypsinogen B have been reported, by Kassell & Laskowski (81), on the chromatographically purified protein before and after performic acid oxidation. No N-terminal amino acid could be detected in either case by the FDNB method, and only after oxidation did carboxypeptidase liberate molar amounts of amino acids (not specified); the thiohydantoin and the hydrazinolysis methods both gave negative results.



**Trypsin.**—Trypsin autolysis has recently been studied rather extensively. By the use of electrophoresis on starch, Liener & Viswanatha (82) were able to separate autolyzed trypsin into residual intact molecules and a less basic component which possessed low activity and was dialyzable. The proteolytic activity of this material, however, was probably due to its interaction with small amounts of undegraded trypsin, as was shown by control experiments. In the presence of calcium ions trypsin could be resolved by this technique into two active components (83), while after urea treatment only one of the components was active. It is interesting to compare this work with that of Chernikhov (84), who found that egg albumin, particularly when denatured, protected trypsin effectively from autolysis. It was shown also that during autolysis the native form of trypsin underwent a degradation to dialyzable end-products which still possessed activity and were stabilized by albumin; consequently, the author is in favor of the existence of "active fragments" of trypsin.

Nord *et al.* (85) have studied the effects of acetylation and addition of calcium ions on the autolytic process. The mode of action of these two protective agents was clarified by kinetic studies and free amino group determinations; the authors also suggested the existence of an enzymatically active degradation product of the native trypsin. These studies on acetyl trypsin have been further extended by Terminiello *et al.* (86) to include other acyl derivatives, with the result that succinyl trypsin was found to be the most stable of all trypsins so far investigated: its self-digestion at about 37° apparently occurred without loss of activity. It is to be noted, however, that at least in the first step, this is also the case for trypsin itself. Wootton & Hess (87) also studied the mechanism of autolysis of acetyl trypsin by determinations of total nitrogen, free amino groups, enzymatic activity and diffusion properties of autolysis products. They came to the interesting conclusion that acetyl trypsin is hydrolyzed primarily by the splitting of all susceptible bonds of one molecule at a time and not through the stepwise hydrolysis of the susceptible bonds of all molecules present (*vide infra*). A marked increase in the specific activity of the enzyme after removal of the cleavage products was apparently observed during these studies.

A study of the essentiality of the disulfide linkages in trypsin by Liener (88) showed that, in the presence of 8 *M* urea, the enzyme was inactivated by concentrations of reducing agents which do not affect its activity in the absence of urea. It was concluded that rupture of the S—S bonds leads to a more extensive, irreversible, unfolding of the molecule, as compared with simple urea-treated trypsin, and this is reflected by a further decrease in the sedimentation constant. The experimental results also indicate that of the three disulfide linkages which become available to reduction under the influence of 8 *M* urea, only one need be split in order to prevent a refolding of urea-treated trypsin and thus to abolish activity. Complementary data are found in studies by Vratisanos *et al.* (89), who have considered the reactions



of trypsin in organic solvents. Trypsin, dissolved in either formamide or dimethyl sulfoxide, immediately loses 50 per cent activity toward hemoglobin, but there is only a negligible loss in esterase activity toward benzoyl arginine ethyl ester, the residual activities being stable in the solvent, except in the presence of added water.

Some data have also appeared concerning the effect of anhydrous acids on trypsin (90). Inactivation was found to follow a first order rate, the process being associated with a surprisingly low entropy of activation. The effect of oxygen on inactivation of trypsin by radiation ( $\alpha$ ,  $\beta$ ) has been discussed by Alexander (91). The oxidation of trypsin and pepsin by periodate has been studied by Maekawa & Tashiro (92).

*Trypsin inhibitors.*—The purity of ovomucoid has been reconsidered by Jutisz *et al.* (93) and a homogeneous antitryptic constituent has been isolated by electrophoresis on a cellulose column.

*Other proteolytic enzymes.*—The inactivation of carboxypeptidase by heat and by surface tension forces was studied in detail by Labouesse (94), who provided kinetic and thermodynamic data for the first process and pointed out the great sensitivity of dilute solutions of the enzyme to the second process, as in lyophilization or even simple dilution. It was found, however, that addition of Tween 80 considerably retarded this latter type of inactivation.

A method for the partial purification of beef spleen cathepsin B has been described (95) as a complement to previous reports (96) on cathepsin C. The preparation obtained appears to be free from other intracellular proteolytic enzymes. Furthermore, it was shown that cathepsin B was activated by sulfhydryl compounds and inhibited by some reagents that combine with —SH groups. The specificity of cathepsin B appears to be directed primarily toward the hydrolysis of amide (or ester) bonds involving  $\alpha$ -N-acylated L-arginine or L-lysine, and in this respect, as already presumed, is quite similar to trypsin. Methods have also been described (97) by which prolidase from swine kidney can be purified considerably and separated from other proteolytic activities known to be present in the organ. The enzyme exhibits a specific requirement for  $Mn^{++}$ , and is stabilized to some extent by glutathione, while it is inhibited by some reagents which combine with —SH groups; the specificity is narrowly directed toward dipeptides such as glycyl-L-proline, and the enzyme may be thus classified as an imidopeptidase.

The nature of the sulfhydryl groups of papain has been discussed recently by Finkle & Smith (98). Previous data have pointed toward the existence in this enzyme of only one reactive —SH group, which is essential for activity. Nevertheless, papain oxidized with performic acid and hydrolyzed yielded six residues of cysteic acid, and denaturation with alcohol led to the appearance of six groups reacting with *p*-chloromercuribenzoate. These data thus suggest the existence of masked —SH groups in papain and also that disulfide bonds are absent from this protein. It should still be noted, as the authors point out,

that these six groups are unable to account for the total sulfur content of papain, which amounts to eight atoms per molecule. As neither methionine, nor inorganic, sulfate nor acid-labile sulfate is present, this point awaits further clarification.

The problem of separating tissue proteases has been considered by Mitz (99), and progress in this direction has been made by the use of cellulose anion exchangers and water-carbon dioxide mixtures as eluents.

As a conclusion to this section, it should be mentioned that the old problem of structural patterns in proteins has been reinvestigated by Šorm *et al.* (100) with particular reference to chymotrypsin and trypsin. On the basis of studies of 68 known structures of proteins or their components, these authors find some regularities, notably a certain selectivity of the bonds between the amino acids, the occurrence of identical peptide sequences in different proteins, and the repetition of structurally related amino acids in peptide chains, suggesting the existence of a certain order in the geometrical arrangement in peptide chains. This appears to be particularly true of proteins that are functionally identical but of different origin, and for proteins which fulfill similar hormonal, enzymic, or other functions in the organism. A set of data are presented (101) in which partial hydrolysates of chymotrypsinogen and diisopropyl phosphoryl-trypsin are compared, and indeed shown to contain a common series of weakly basic di- and tripeptides.

#### KINETICS

*Chymotrypsin.*—Detailed kinetic studies on the reactions of  $\alpha$ -chymotrypsin with synthetic substrates have been extensively pursued by Niemann's group. In the case of the hydrolysis of acetyl-L-tyrosinamide and of acetyl-L-tryptophanamide (102), it was found, under the conditions used (25°, pH 7.90) that the values of  $K_s$ ,  $k_2$  and  $K_p$  for the acetyl-L-tyrosinamide system, obtained by observing the rate of formation of either one of the two reaction products, were identical. Furthermore,  $K_s$  and  $K_p$  were independent of the nature and concentration of the buffers used (TRIS, ethylenediamine, phosphate), while  $k_2$  was dependent on the concentration of the buffer components and, indirectly, upon their nature. More limited observations with acetyl-L-tryptophanamide suggest that its behavior is similar to that observed for the tyrosine analogue. Use of the same ninhydrin method as in the previous study allowed the authors (103) to re-examine further a discrepancy between previous values (104, 105) and the above-mentioned ones for the enzyme-inhibitor dissociation constant,  $K_p$ , of the two bifunctional anionic competitive inhibitors of  $\alpha$ -chymotrypsin,  $\beta$ -( $\beta$ -indole)-propionate and phenylacetate. A detailed analysis showed indeed that the lower values obtained by the earlier authors may be increased by lowering the concentration of the univalent buffer system used, or by reducing the amount of sodium chloride or potassium phosphate present, the effect being associated with a

general decrease in ionic strength as well as with the influence of the specific ions.

Martin & Niemann (106) have also observed that the initial rate of the  $\alpha$ -chymotrypsin catalyzed hydrolysis of methyl hippurate in aqueous solution at 25°, pH 7.90, was markedly dependent upon the presence of added sodium chloride. At concentrations of sodium chloride greater than 1 *M*, the value of  $K_s'$  was essentially constant, but below 1 *M*,  $K_s$  increased and asymptotically approached infinity at zero salt concentration. Conversely,  $k_3'$  continually decreased and approached zero at zero salt concentration. This phenomenon may be due to change in property of the enzyme molecule, which may be transformed into a species which is incapable of combining with a specific substrate. Alternatively, these results may be interpreted in terms of an effect of ionic strength on the catalytic step *per se*.

In view of their possible spectrophotometric determination, the usefulness of a series of hydrazides of  $\alpha$ -amino acids and acylated  $\alpha$ -amino acids as specific substrates for studies involving  $\alpha$ -chymotrypsin was also considered by Lutwack *et al.* (107). The pH optimum (25°C.) was found to be  $7.05 \pm 0.15$  for the hydrolysis of L-tyrosinehydrazide, and approximately 7.7 to 8.0 for the comparable reactions of six  $\alpha$ -N-acylated-L-tyrosinehydrazides; the values pertaining to this last group of substrates, however, were strikingly temperature-dependent in the region between 25 and 40°C., an increase in temperature causing the pH optima to shift to a more acidic region.

In a comparison of the action of chymotrypsin on denatured lysozyme in solution and at a solid-liquid interface (as adsorbed on kaolinite), McLaren & Estermann (108) showed that the pH optimum is narrower and shifted to a higher value in the latter case, as could be predicted on the basis of the ionization properties of the kaolinite surfaces. The rate of chymotryptic catalysis has also been the subject of a potentiometric study described in a thesis by Lang (109), while the esterolytic properties of proteinases (chymotrypsin, trypsin, and papain) have been considered in a dissertation by McDonald (110).

**Trypsin.**—The hydrolysis of benzoyl-L-argininamide by trypsin was studied kinetically by Shukuya & Watanabe (111); it was shown that the reaction does not follow the conventional first order kinetics. From the integrated form of Michaelis & Menten's equation, a value for  $k_3$  was thus obtained, independent of both enzyme and substrate concentration, while  $K_m$  was determined from a Lineweaver-Burk plot. The temperature dependence of the reaction allowed heats of enzyme-substrate complex formation and of activation to be calculated as 5.9 and 12.3 kcal./mole (mol. wt. 17,000), respectively. The existence of a group with  $pK' = 6.28$  at 30°C. and having a heat of ionization = 7.1 kcal./mole was found to be in agreement with the pH dependence of the reaction, a finding which is in accord with the data of Gutfreund (112) for the tryptic hydrolysis of benzoyl-L-arginine ethyl ester, and which is thus susceptible to the same interpretation, namely, the involve-

ment of an ionizable group, possibly histidine, in the reaction. On the other hand, the hydrolysis of benzoyl-L-arginine ethyl ester itself, in the presence of dioxane, has been further considered by Inagami & Sturtevant (113). No essential change in the nature of the reaction was detected in comparison with the data obtained by Gutfreund (above) in purely aqueous solutions; hence the deviations in mixed solvents can probably be interpreted in terms of the effect of the dielectric constant of the medium on charge interaction, and of the activity of the water in the mixed solvents.

The kinetics of the reaction between trypsin and the pancreatic trypsin inhibitor have been thoroughly studied by Green (114) as a function of temperature and ionic strength at pH 7.8, using a potentiometric method of high sensitivity. Variation in temperature allowed values for the heat and entropy of activation to be calculated, while the effect of ionic strength on the rate at 25°C. was found to be in accord with the Brønsted-Bjerrum theory for reactions between ions of like charge. Furthermore, data presented for the dissociation of the inhibitor-trypsin compound at acid pH are consistent with a reaction scheme in which the combination between inhibitor and trypsin is dependent on the ionization of three specific carboxyl groups. The role of electrostatic forces in this reaction is discussed in detail in terms of current theories of protein interaction, with the conclusion that although electrostatic repulsion in alkaline medium cannot account for the observed kinetic constants, it may well explain the low rate of this combination relative to that of the other inhibitors.

Mutual precipitation of trypsin and heparin was investigated by Mansfeld & Hladovec (115) and shown to yield, at pH's lower than 8.4, a stable adduct having both proteolytic and anticoagulant activities, and of constant composition irrespective of the ratio of the reacting compounds, the reaction reaching a maximum at pH 4. The adduct of heparin with chymotrypsin was similarly prepared.

*Aminopeptidase.*—The study of the mechanism of action of leucine aminopeptidase on its substrates has been pursued by Hill & Smith (116) with the use of specific inhibitors. The authors were able to show that not only metal chelating agents, as previously described (17), but also aliphatic alcohols, and better, aliphatic amino alcohols or aliphatic acid amides, are competitive inhibitors for leucine aminopeptidase; the most effective one found thus far was  $\alpha$ -hydroxyisocaproamide, which is a poor substrate as well. Thus, the present studies lend additional support to the concept of a hydrophobic type of interaction at the specificity site, and to the fact that, in contrast to the behavior of certain other proteolytic enzymes, e.g., trypsin, chymotrypsin, carboxypeptidase, and papain, all of which possess esterase activity, leucine aminopeptidase interacts with the peptide or amide nitrogen of the sensitive bond rather than with the carboxyl group of this bond. This conclusion was also supported by the fact that no transferase action could be demonstrated with leucine aminopeptidase.

The action of this enzyme on long-chain polypeptides and proteins also has recently been considered by Hill & Smith (117). Oxidized A and B chains of insulin were found to be readily hydrolyzed by aminopeptidase, and kinetic analysis of the amino acids liberated, allowed a determination of the N-terminal sequences of these peptides, which, in the case of the B chain was consistent with its known structure, while with the A chain experimental difficulties complicated the interpretation. Zinc-insulin, native serum albumin, ribonuclease, and lysozyme were also investigated and found resistant in the native state, probably as a result of hydrogen bond or disulfide stabilization, but in general their performic oxidized derivatives were readily degraded. All the results obtained showed complete absence of endopeptidase activity in highly purified leucine aminopeptidase; it was further observed that the enzyme was only partly and reversibly by concentrated solutions of urea and acetamide, and irreversibly inactivated by guanidine hydrochloride.

*Other proteolytic enzymes.*—Some kinetic work has also appeared on a partially purified preparation of beef spleen cathepsin C (118), which was examined with respect to the pH dependence of its hydrolytic action on L-prolyl-L-phenylalaninamide (optimum near pH 7), the effect of several activators (cysteine,  $\beta$ -mercaptoethanol and 2,3-dimercaptopropanol) and also the rate of the hydrolysis of a series of structurally related dipeptide amides and dipeptide esters which serve as substrates or as inhibitors. These data suggest that the enzyme-substrate combination involves the interaction of several groups with possible hydrogen bonding in a manner similar to that demonstrated for trypsin and chymotrypsin (119).

The kinetics of the hydrolysis of benzoyl-L-argininamide by papain have also been the subject of a very detailed study by Stockell & Smith (120). The determination of the kinetic parameters  $K_m$  and  $k_3$  under a wide variety of conditions, including changes in pH, temperature, dielectric constant and presence of  $D_2O$ , was described, leading to evidence which supports the interpretation, in most cases, of  $K_m$  as the stationary rate constant  $k_3/k_1$ , a fact which then allows an estimation of  $k_1$ . From the values obtained for this latter constant as a function of pH, it was inferred that the first step of the reaction requires the presence of two titratable groups, probably an ionized  $\beta$ -carboxyl group and an unionized sulfhydryl group, at the active site of the enzyme; data concerning the inhibition of the reaction by carbobenzoxy-L-glutamic and carbobenzoxy-L-aspartic acids were shown to be consistent with the proposed nature of the first step. An expression was also derived for the over-all velocity of the reaction by assuming that the rate constant  $k_3$  actually represents a combination of two rate constants of comparable magnitude involved in two successive breakdown steps; thereby, some apparently aberrant experimental results could be explained. A tentative mechanism was presented on the basis of the preceding conclusions, its main feature being, as already suggested earlier (121), the formation of a thiol ester in the enzyme substrate complex and its subsequent hydrolysis by attack of

OH<sup>-</sup> ions. This work, together with that on aminopeptidase, represents a most valuable addition to the study of the mechanism of enzyme action, and the conclusions are particularly interesting in relation to similar findings reported elsewhere in this chapter, concerning the mode of action of other esterase-type proteolytic enzymes.

*Methods.*—The conversion of ovalbumin to plakalbumin by subtilisin, the main characteristics of which were described several years ago (122), was followed kinetically by Ottesen (123, 124) in the pH-stat (125). Confirming previous suggestions by Steinberg (126), the reaction was found to consist of at least two steps, the first one being the opening of a single peptide bond in a closed chain of the ovalbumin molecule, leading to the formation of an open-chain, intermediary protein which had lost the ability of crystallizing in needles. In a further reaction which remains to be elucidated, this form is converted into the more soluble plakalbumin. A micro-colorimetric method for the determination of amino acids, peptides and proteolytic activity was described by Rademaker & Soons (127), who could show kinetically that formation of an enzyme-cobalt complex initiates the hydrolysis of glycylglycine by rat liver dipeptidase. Other improvements in the field of methodology of proteolysis rate studies include the following: Christensen (128) has demonstrated the capacity of strong urea solutions to dissolve TCA precipitates, this serving as a basis for methods of determining the proteolytic activity of pepsin and trypsin. A manometric method has been devised (129) for following peptidase activity, while a new quantitative determination of the activity of pepsin has been described by Krówczyński & Smajkeiwicz (130) based on the diffusion of the enzyme into agar-casein gels. The method was used for determination of the proteolytic activity of gastric juice. The determination of slow proteolysis rates was considered by Pantlitschko & Gründig (131), who developed a modified biuret reaction useful for the estimation of trypsin, pepsin, and thrombin at the microgram level. A rapid and sensitive method has been developed by Hess *et al.* (132) which allows the accurate determination of proteolytic enzyme concentrations at very low levels by the use of an insoluble protein dye complex as substrate. Loseva (133) has described the stabilizing action on pepsin of protein hydrolysates obtained by the action of pepsin, papain, and pancreatin, and which is probably due to the inhibiting action exercised by peptides of low molecular weight on the autolysis of the enzyme.

#### SPECIFICITY

The action of various proteolytic enzymes on peptides and derivatives containing histidine has been investigated by Davis (134). Leucine aminopeptidase was found to hydrolyze L-histidinamide and L-histidyl peptides, but not histidine peptides. Carbobenzoxylglycyl-L-histidine was found to be a poor substrate for carboxypeptidase, despite the ring structure in the C-terminal amino acid, whereas carbobenzoxyl-L-histidyl-L-phenylalanine and



the tyrosine analogue were found to be rapidly hydrolyzed by this enzyme. Carnosinase (swine kidney) hydrolyzed at approximately equal rates the peptides carnosine, anserine,  $\beta$ -alanyl-L-histidylglycine and histidylglycine. Histidyl peptides were generally poor substrates for papain and for chymotrypsin, carbobenzyloxyl-L-histidinamide being hydrolyzed at 3 per cent of the rate of carbobenzyloxyl-L-tyrosinamide. The synthesis of a number of peptides and peptide derivatives containing histidine and tryptophan has been described as part of this investigation.

A number of *n*-fatty acid esters of meta- and ortho-hydroxybenzoic acids were found by Hofstee (135) to be substrates for typical esterases, such as trypsin and chymotrypsin; these are structurally analogous to the acid anhydrides and acid chlorides which in the investigations of Dixon & Neurath (136) served as substrates for chymotrypsin (*vide infra*). With trypsin, maximum hydrolysis was observed when there were six carbons in the fatty acid moiety of the substrate, and with chymotrypsin, seven carbons. Enzymatic hydrolysis was inhibited by typical inhibitors for these enzymes, such as soybean trypsin inhibitor and  $\beta$ -phenylpropionate.

Knowledge of the specificity of well-characterized proteolytic enzymes has also been extended by various other investigations which are quoted below.

**Chymotrypsin.**—Whereas Sanger & Thompson (137) reported that  $\alpha$ -chymotrypsin hydrolyzes a peptide bond between cysteic acid and serine in the A chain of bovine insulin, Mower & Niemann (138) found that N-acetyl-L-cysteic acid carboxamide is resistant to chymotryptic hydrolysis within the pH range 6.2 to 8.3 at 25°. Since the same compound failed to cause competitive inhibition in the chymotryptic hydrolysis of the specific substrate,  $\alpha$ -N-acetyl-L-tyrosine hydrazide, it was concluded that the lack of hydrolysis of N-acetyl-L-cysteic acid carboxamide by chymotrypsin was most probably the result of a lack of combination with the catalytically active site of the enzyme. Since, in the A chain of insulin, three other cysteic acid peptide bonds were inert toward chymotrypsin, the authors suggest that the susceptibility of this particular peptide bond could have arisen, at least in part, from an N $\rightarrow$ O acyl migration (139, 140) during the preparation of the A chain of insulin, the resulting ester being more susceptible to chymotryptic hydrolysis than the peptide. N-methyl and -ethyl derivatives of phenylalanine ethyl ester were found by Kuk-Meiri & Lichtenstein (141) to be entirely resistant to both hydrolysis and transpeptidation by chymotrypsin. These negative findings were considered to support the hypothesis that a secondary peptide bond is an essential structural requirement for specific substrates (119). The authors suggest that the previous findings by Goldenberg & Goldenberg (142), according to which phenylalanine ethyl ester was readily hydrolyzed by chymotrypsin, could be more adequately explained by assuming that chymotrypsin cannot hydrolyze these esters as such, but that by a process of transpeptidation higher peptides are formed which are subsequently hydrolyzed by the enzyme.



**Trypsin.**—Weil & Telka (143) have provided convincing confirmatory evidence for the exclusive specificity of trypsin toward arginyl and lysyl peptide bonds. Thus, whereas in native  $\alpha$ -lactalbumin 13 bonds were hydrolyzed by trypsin, in N-acetyl  $\alpha$ -lactalbumin (with all 12  $\epsilon$ -amino groups and the single  $\alpha$ -amino group acetylated) only 1.5 bonds were hydrolyzed (because of the single arginyl bond in the molecule). Similarly, in fully guanidinated  $\alpha$ -lactalbumin, wherein the lysyl groups were converted to homo-arginyl groups, and in fully dinitrophenylated ( $\alpha$ ,  $\epsilon$ )  $\alpha$ -lactalbumin, only 1.5 bonds were split by trypsin. In the light of these findings, the extensive fragmentation arising during "autolysis" of acetylated trypsin, reported by Liener & Wang (144), must be ascribed to the action of enzymes other than trypsin (*vide infra*), particularly since C-terminal groups (lysine and arginine) arising from tryptic hydrolysis would not be removed by carboxypeptidase (Anson). In a study of the synthesis and enzymatic hydrolysis of poly-D-lysine, Tsuyuki & Stahmann (145) found that this polymer was not hydrolyzed by trypsin, whereas the corresponding L-isomer was degraded to the extent of approximately 20 per cent. Some interaction between poly-D-lysine and trypsin must occur, however, as the D-isomer completely inhibited the tryptic hydrolysis of the L-isomer. Pancreas powder, however, hydrolyzed both isomers equally well, to the extent of approximately 90 per cent. Various optical isomers of the tripeptide, alanyl-lysyl-alanine, were tested by Clark & Ellenbogen (146) as substrates for trypsin. The initial rates of hydrolysis decreased in the order of LLD, LLL, DLD and DLL, the corresponding turnover numbers being 4200, 2800, 800 and 80. The authors suggest that the specificity requirements of trypsin be redefined to include the provision that an L-amino acid be attached to the  $\alpha$ -amino group of the central amino acid residue.

Although the subject of enzymes involved in blood clotting has been omitted from this review, it should be mentioned that, in addition to arginyl esters, thrombin has now been found by Ehrenpreis *et al.* (147) to hydrolyze lysine ethyl ester also; but, in contradistinction to trypsin, the hydrolysis of these compounds by thrombin is not inhibited by soybean trypsin inhibitor. The authors also report that the B chain of insulin is hydrolyzed by thrombin at the lysyl-alanine bond.

A new synthetic substrate for trypsin has been described by Lindley (148) namely, the polymer of S-( $\beta$ -aminoethyl)-L-cysteine, which may be said to be an analogue of lysine in which a methylene group is replaced by a sulfur atom. Tryptic hydrolysis, when followed by paper chromatography, was found to yield a number of as yet unidentified degradation products, probably consisting of the amino acid derivative and small polymers thereof. The possible application of these observations to the determination of the C-terminal cystine groups in proteins is evident.

**Pepsin.**—Neuhaus & Miller (149) compared acetylated and non-acetylated ovalbumin toward peptic digestion. It was found that native and N-acetyl ovalbumin were more readily hydrolyzed by pepsin at pH 1.6 than

N,O-acetyl ovalbumin. Neumann *et al.* (150), from Katchalski's laboratory, have studied the pepsin-catalyzed hydrolysis of N-acyl dipeptides containing tyrosine or phenylalanine or both. Chromatographic analysis of the products of the reaction led the authors to the conclusion that transpeptidation as well as hydrolysis occurs, the transpeptidation reaction involving an amine rather than a carboxyl transfer (151), the unionized carboxyl group serving as the acceptor.

*Papain.*—Mycek & Fruton (152) have extended their studies on the papain-catalyzed transfer reactions, and have found a pronounced influence of the  $pK_s'$  of the dipeptide on the efficiency of its participation in transamidation reactions. In these studies, the transpeptidation between carbo-benzoxymethylglycinamide and a series of dipeptides was studied by chromatographic isolation of the product of the reaction. Whereas the configuration of the C-terminal amino acid (L vs. D) had little influence on the extent of transamidation, that of the N-terminal amino acid was profound. In general, leucyl peptides were more effective reactants than glycyl peptides, and leucyl-L-phenylalanine or -tyrosine more efficient than leucyl-L-leucine. Johnson & Herriott (153) have confirmed and extended the demonstration of Behrens & Bergmann (154) of the existence of "cosubstrates" of proteolytic enzymes. Thus, whereas glycylglycinamide is resistant to cysteine-activated papain, in the presence of glycyl-L-leucine, acetyl-DL-phenylalanylglycine, acetyl-DL-phenylalanylalanine, benzoyl-DL-leucylglycine,  $\delta$ -aminovaleryl-phenylalanylglycine, or glutathione, hydrolysis occurred. Whereas several monoamino-monocarboxylic, di- and tripeptides did not function as cofactors if the free amino group was in the  $\alpha$  position, the amino group was found to confer cofactor activity if in the  $\delta$ -position. Purified horse serum albumin and acid-washed casein likewise conferred cofactor activities. Since by paper chromatography, no intermediate peptides could be demonstrated, transpeptidation seems to be excluded as a mechanism of these reactions. Tollin & Fox (155) have described competition experiments between "carboxoid" reactants in papain-catalyzed reactions with glycylglycinamide. The relative reactivities of the benzoyl amino acids or benzoyl dipeptides with glycylglycinamide were found to increase in the order of benzoyl-DL-alanine, benzoyl-glycyl-DL-valine, benzoylglycine, benzoyl-DL-leucylglycine and benzoyl-DL-leucine. In a succeeding paper of this series (156), these authors reported on studies of the effects of cysteine hydrochloride and various buffer components on the yield of the papain-catalyzed reaction between benzoylglycine and glycylglycinamide.

*Rennin.*—The substrate specificity of crystalline rennin appears to be similar to that of pepsin (157). Using the B chain of insulin as substrate, rennin was found to split the following bonds: -leucyl-valyl-, -leucyl-tryosyl-leucyl-, phenylalanyl-phenylalanyl-tyrosyl. In contrast to chymotrypsin, which also splits some of these bonds, rennin does not liberate the peptide threonyl-prolyl-lysyl-alanine from the B chain of insulin, nor does it split

acetyl-L-tyrosine ethyl ester. The pH optimum of crystalline rennin is pH 4, in contrast to pH 2 for pepsin.

*Carboxypeptidase*.—Yanari & Mitz (158) have previously described the hydrolysis of unsubstituted dipeptides by carboxypeptidase. In a recent study (159), it was found that the acyl moiety of the dipeptide exerts a modifying influence on the susceptibility of the peptide to hydrolysis by pancreatic carboxypeptidase. Thus, whereas D-leucyl-L-tyrosine and L-leucyl-L-tyrosine were hydrolyzed at the same rate, acylation of L-leucyl-L-tyrosine increased the rate 50- to 200-fold, depending on the nature of the acyl substituent, whereas D-leucyl-L-tyrosine, on acylation, became resistant to carboxypeptidase action. These studies are in accord with previous observations (119) on the importance of the secondary peptide bond for the rapid hydrolysis of peptides by carboxypeptidase. In a companion investigation, Yanari & Mitz (160) found that dipeptides were inhibitors for carboxypeptidase, the anionic form with an uncharged amino group being the inhibitory species. Values for  $K_i$  were determined from Michaelis-Menten equations, modified to include the inhibition (at pH 9.0) both by the anionic species of the dipeptide and by the product of hydrolysis, i.e., the C-terminal amino acid of the substrate. It is of considerable significance that, according to the data, the order of hydrolytic susceptibility is quite different from that of affinity. Thus, the low hydrolytic rates of the dipeptide cannot be ascribed merely to a low affinity for the enzyme, since the poorest unsubstituted dipeptide substrate formed the most stable complex with carboxypeptidase. Liener & Wang (144) found that carboxypeptidase liberates approximately 35 amino acids from acetylated trypsin, this being, presumably, due to C-terminal groups arising from self-digestion (*vide supra*). After 24 hr. of autolysis, 50 per cent of the nitrogen of acetylated trypsin became TCA-soluble, with no detectable loss in enzymatic activity (using casein and benzoyl arginine methyl ester as substrates). It is reported, in this preliminary communication, that separation into active and inactive components could be accomplished by starch electrophoresis, and that the active fraction had a 50 per cent greater specific activity (on a nitrogen basis) than the original enzyme. The action of carboxypeptidase on crystalline fructose diphosphate aldolase was described by Drechsler (161). After liberation of three equivalents of tyrosine and some threonine, the enzyme had only 7 per cent of the original activity toward fructose-1,6-diphosphate, whereas the aldolase activity toward fructose-1-phosphate, which was initially low, was increased three-fold during this reaction. Degradation by carboxypeptidase had no effect on the ultracentrifugal behavior of the enzyme.

#### THE ACTIVATION OF ZYMOGENS

Since the problem of zymogen activation has been the subject of a recent comprehensive review (5), only the latest and most pertinent contributions to this subject will be considered herein.

The activation of trypsinogen by trypsin has recently been studied in considerable detail (4, 162) by comparing, with the appearance of enzymatic activity, (a) the release of protons resulting from the splitting of peptide bonds, as determined by titration in the pH-stat; and (b) the amount of trypsin appearing, as determined by electrophoretic analysis of activation mixtures. When titrations were carried out at pH 8, in the presence of indole (to minimize hydrolysis resulting from the presence of a chymotrypsin-like enzyme in trypsinogen),<sup>3</sup> the data could be interpreted in terms of the liberation of one or two equivalents of protons released per mole of trypsinogen, depending on the pK value assumed for the  $\alpha$ -amino group. The percentage change in hydrogen ions appearing with time followed the same rate curve as the appearance of enzymatic activity; and since the points describing the percentage change of appearance of the hexapeptide, valyl-aspartyl-L-lysine, also followed the activity curve, it appears likely that no peptide bond other than that giving rise to the appearance of the hexapeptide had been broken during activation. Further, when both parameters were plotted on a percentage basis, the rate of appearance of trypsin (as trypsin-inhibitor compound), as measured electrophoretically at pH 8 in the presence of an excess of soybean-trypsin inhibitor, also followed the rate curve for the appearance of enzymatic activity. These experiments considerably strengthen the view that the primary event in the autocatalytic activation of trypsinogen is the splitting of a uniquely positioned lysine-isoleucine bond, which then gives rise to the liberation of the hexapeptide, and to such changes in configuration as are responsible for a decrease in the levorotation (164) and the appearance of enzymatic activity.

A unique structural feature of trypsinogen, trypsin, and chymotrypsinogen is the apparent absence of a reactive C-terminal group. Thus, all three proteins fail to yield free amino acid upon hydrazinolysis and are likewise unreactive toward carboxypeptidase or "basic" carboxypeptidase (24). Although Folk, Gladner & Laki (165, 166) have reported that crystalline trypsin, upon incubation with "basic" carboxypeptidase, yields lysine as a reaction product, this does not occur when trypsinogen, before or after activation is tested in a similar manner (162), which suggests that further (autolytic) changes have accompanied the formation of crystalline trypsin from fully activated trypsinogen. C-terminal structures which would not yield free amino acids by any of the above-mentioned methods include (a) those terminating in an  $\alpha$ -amide, (b) a C-terminal half-cystine residues, or (c) structures wherein the  $\beta$  or  $\gamma$  carboxyl of C-terminal aspartic or glutamic acid or the  $\alpha$ -carboxyl group of any C-terminal group is involved in peptide bond forma-

<sup>3</sup> The observation that trypsinogen may contain appreciable amounts of a chymotrypsin-like enzyme appears to militate against the contention of McFadden & Laszkowski (163) that the low activity of trypsin toward chymotrypsin substrates is a property of trypsin itself, rather than being due to a contaminating trace of chymotrypsin in trypsin.

tion with other side chains in the molecule. No experimental data have yet been published to test or exclude any of the aforementioned hypothetical configurations.

While in the presence of calcium ions, at pH 8, the conversion of trypsinogen to trypsin is quantitative, in the absence of calcium ions, trypsinogen may be converted, in part or *in toto*, to a protein devoid of enzymatic activity, i.e., the "inert protein" first described by Kunitz (167). The molecular changes accompanying this reaction have recently been partly elucidated by Desnuelle & Gabelot (168). These authors found, in agreement with previous work (169), that, in the presence of calcium ions, activation of trypsinogen (0°, pH 7.8) was complete. However, in the absence of calcium a limiting value of approximately 50 per cent of maximum potential activity was observed, which could be shown to be due to the conversion of some of the trypsinogen into "inert" protein, rather than being due to autolytic destruction of any initially formed trypsin. Since analysis of the peptide fraction of activation mixtures showed that, in the absence of calcium, only half as much of the hexapeptide was formed, it was concluded that the conversion of trypsinogen to "inert" protein was not accompanied by the splitting of the lysyl-isoleucine bond. This was further substantiated by analyses of N-terminal groups of the protein fraction of activation mixtures prepared in the presence and absence of calcium ions, respectively. Thus, in the presence of calcium ions, in agreement with earlier work (170), 0.9 mole of DNP-isoleucine was obtained, in addition to insignificant amounts of DNP-valine; in the absence of calcium ions 0.5 mole (presumably of each) of DNP-isoleucine, DNP-valine, and DNP-serine were found. The first of these was attributed to the trypsin formed, whereas DNP-valine and DNP-serine were attributed to N-terminal groups of "inert" protein, the former being identical to that in trypsinogen, and the latter arising from a hydrolytic step not involved in the formation of trypsin. These results lend weight to the view that calcium exerts a specific effect in the formation of trypsin by directing the hydrolytic step to the peptide bond which gives rise to the liberation of the hexapeptide, whereas in the absence of calcium ions other peptide bonds elsewhere in the single chain of the trypsinogen molecule are attacked, leading to inert protein.

A schematic representation incorporating all known events involved in the activation of trypsinogen has been recently proposed by Neurath & Dixon (4) and is reproduced herein (Fig. 1). It is proposed that the N-terminal region of the molecule, containing four adjacent aspartic acid side chains, does not fit into a helical configuration, and that interaction between the aspartyl carboxyl groups, electrostatically with positively charged groups or by hydrogen bonding with other groups elsewhere along the chain, provides a loop which is destroyed when the lysyl-isoleucine bond is broken by trypsin. The N-terminal region of the molecule then reorients itself so as to establish the interaction of a histidine and a serine side chain (*vide infra*),





the latter likely to be part of a rigid structure which contains four of the twelve half-cystine groups.

Recent work on the slow activation of chymotrypsinogen (69, 171) suggests that in the conversion of chymotrypsinogen to  $\alpha$ -chymotrypsin, two and only two dipeptides are split off. One of these is serylarginine, previously found as a product of the rapid activation of chymotrypsinogen (172, 173), and the other one, threonylasparagine. The problem of the interrelation in primary structure of chymotrypsinogen and the products of rapid and slow activation has been considerably clarified by studies of Desnuelle *et al.* (69), who were able to show that the sequence of events in the formation of  $\alpha$ -chymotrypsin could be reversed by allowing chymotrypsinogen to react first with chymotrypsin and then with trypsin. While complete separation of the three new forms of chymotrypsinogen from one another has apparently not yet been achieved, the various neo-chymotrypsinogens have been partly characterized by end-group analysis. The formulations shown in Figure 2 represent the current state of knowledge of this subject.

Evidence for changes in secondary or tertiary structure during the activation of chymotrypsinogen and trypsinogen has been recently obtained (164) by measurements of the optical rotation of activation mixtures. A decrease in specific levorotation on activation of these zymogens could be correlated with the appearance of enzymatic activity, suggesting that in both instances the activation leads to a more nearly helical configuration. Comparison of the rate of change of optical rotation, viscosity, and biological activity of chymotrypsinogen and  $\delta$ -chymotrypsin in urea has indicated that these parameters do not change in precisely the same manner. Despite the extensive changes in configuration which occur in urea solution, both chymotrypsinogen and  $\delta$ -chymotrypsin were found capable of regaining the active configuration when urea was removed. These observations appear to be partly at variance with similar studies by Harris (174), although slightly different experimental conditions were observed by that author. These investigations, in conjunction with other experimental evidence, have led to a picture of the activation of chymotrypsinogen which involves the formation of one or more unstable regions during activation; these regions are more sensitive to changes in urea concentration and pH, in contrast to more stable regions which are unaffected by activation and are common to both chymotrypsinogen and  $\delta$ -chymotrypsin. Additional evidence for intramolecular changes occurring during the activation of chymotrypsinogen has been recently provided by measurements of the differential absorption spectrum in the ultra-violet region. Thus, it was found by Chervenka (175) that the percentage change in absorption at 288  $m\mu$  occurring during the rapid activation of chymotrypsinogen (under conditions leading predominantly to  $\pi$ -chymotrypsin) followed exactly the curve which describes the appearance of enzymatic activity. Moreover, the difference spectrum between chymotrypsinogen and chymotrypsin, at pH 7.8, is of a type usually associated with a rupture of hydrogen bonds involving the hydroxyl groups of tyrosine side chains.



Previous studies of the activation of procarboxypeptidase, by Keller, Cohen & Neurath (176) have now been extended (177), using a preparation of the zymogen obtained by chromatographic resolution on diethylaminoethyl-cellulose ion exchange resin. The unique observation was made that purified procarboxypeptidase appears to be the zymogen both for carboxypeptidase and for an endopeptidase which hydrolyzes acetyl-L-tyrosine ethyl ester. Conditions for activation of the two enzymes are quite different, the formation of the endopeptidase being catalyzed by low concentrations of trypsin, whereas the formation of carboxypeptidase involves the participation of both the endopeptidase and of relatively high concentrations of trypsin. Further, whereas the endopeptidase seems to have the same molecular weight as procarboxypeptidase itself, carboxypeptidase activity is associated with the core, comprising only one-third of the molecular weight of the zymogen. The synergistic effect of the two activities inherent in procarboxypeptidase preparations is particularly striking, as both activities are primarily directed toward those residues which contain an aromatic ring in the side chains. The endopeptidase thus produces new carboxyl terminal groups of the very configuration which favors hydrolysis by carboxypeptidase.

The structural relations between pepsinogen and the products resulting from its activation have been recently considerably clarified by Van Vunakis & Herriott (53, 54) and are considered elsewhere in this review.

#### ACTION OF PROTEOLYTIC ENZYMES ON PROTEINS

As has been mentioned in the introduction, detailed discussions of the action of proteolytic enzymes on the primary structure of proteins are available elsewhere. In this section, therefore, only recent work dealing with the influence of secondary and tertiary structure will be considered. One main pattern has been made clear in the past; disoriented or denatured protein, which, presumably, is largely in the form of randomly coiled peptide chains, is much more rapidly attacked by proteolytic enzymes than the native protein (122). Sri Ram & Maurer (178) have studied the proteolysis of a number of chemical modifications of serum albumin produced by acetylation, guanidination, deamination, etc., and in almost all cases found that these modifications were attacked much faster by proteolytic enzymes than the native molecule; it is concluded that the chemical modification itself, or the conditions of the modification, produced partial denaturation of the albumin. In the case of the action of trypsin on albumin, even acetylation, which would be expected to decrease the number of trypsin-susceptible peptide bonds, increased the rate of proteolysis; thus, the increase in rate due to partial denaturation obscured any decrease due to acetylation of the  $\epsilon$ -NH<sub>2</sub> of lysine residues. In many cases the increased susceptibility of the modified proteins to proteolytic attack could be correlated with their partial denaturation, using physico-chemical criteria such as solubility, etc.

In a detailed and critical series of papers, Labeyrie (179, 180) has re-

ported on studies of the proteolysis of native and heat-denatured  $\beta$ -lactoglobulin. Initially, a study (179) of the methods of measurement of proteolysis showed that while the titration of newly-appearing free carboxyl groups [Linderstrøm-Lang method (122)] corresponded closely to the appearance of amino groups by potentiometric titration at constant pH, the TCA method gave results which were not in close agreement with the above. This was shown to be due to the adsorption onto the insoluble protein precipitate of soluble material absorbing light at 280  $m\mu$ . In measurements of the kinetic constants for the hydrolysis of native and two different heat-denatured forms of  $\beta$ -lactoglobulin by trypsin (180) it was found that the enzyme had an affinity for the denatured form which was three times that for the native. The rate constant  $k_s$  was found to vary with temperature in a different manner for the native and denatured protein, the energies of activation being 18,600 cal./mole for native and 14,200 cal./mole for denatured proteins. From these data, the changes of entropy and enthalpy of activation were calculated according to the absolute rate theory. The results were interpreted as favoring the view that the limiting process of the over-all rate of proteolysis corresponds in the native protein to a simultaneous breaking of a peptide linkage and three of four hydrogen bonds, these four hydrogen bonds being responsible for keeping one loop of an  $\alpha$ -helix in place. In the same laboratory, Yon (181) has determined the  $pK$ 's of the new amino groups arising during the tryptic and chymotryptic degradation of native and denatured  $\beta$ -lactoglobulin; values which ranged from 6.37 to 7.40 were found.

Another aspect of proteolysis that has received some attention in the last year is the question of whether protein molecules are attacked "one by one" and reduced to the final products, or whether all protein molecules are progressively attacked, there being a series of intermediates between unattacked protein and final products present in the reaction mixture (182). Ginsburg & Schachman (183) have defined the problem as a question of the rate of splitting or "susceptibility" of the first bond in the protein compared with the rates of splitting of subsequent bonds. The limiting cases would fall into the two categories mentioned above: if the first bond were split very rapidly as compared with subsequent ones, then probably this bond would be split in all substrate molecules before other bonds, particularly when the enzyme concentration was high. On the other hand, if the first bond were split very slowly compared with subsequent ones, the complete degradation of one molecule at a time would be likely. It is probable that different combinations of substrate and enzyme fall at different places in the "spectrum" between these two limiting cases. The above authors have studied the degradation of insulin by chymotrypsin by using the "synthetic boundary" cell in the ultracentrifuge and have seen no intermediates; thus the proteolysis would belong to the first type. They were also able to show by end-group analysis that the same peptide bonds were broken in native insulin as in the separated chains after oxidation. On the other hand, during the action of pepsin on ribonuclease, the molecular weight decreased slowly at first and

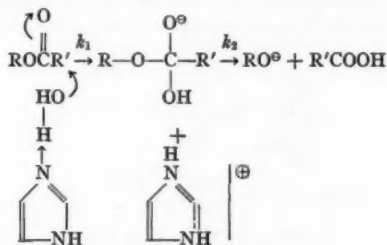
### Reaction Sequence I

Thus Bender & Turnquest (188) have shown that the formation and disappearance of a species which absorbs at  $E_{245}$  (corresponding to N-acetyl imidazole) can be demonstrated.

More recently, Brouwer *et al.* (190) have demonstrated this intermediate in the imidazole-catalyzed hydrolysis of acetic anhydride, *p*-nitrophenylacetate, and phenylacetate, and have shown that its concentration reaches a high enough level to account for the entire hydrolysis in the initial stages.

Brecher & Balls (191) have observed a similar intermediate in the hydrolysis of *p*-nitrophenylacetate catalyzed by a  $\alpha$ -N-benzoyl histidine methyl ester, and have isolated the  $\alpha$ -N-benzoyl-1- or 3-N-acetyl histidine methyl ester as an intermediate. Bernhard & Gutfreund (192) have also demonstrated the appearance of an  $E_{245}$  intermediate with histidine methyl ester.

Bruice & Schmir (189) have carried out similar studies and postulate a mechanism similar to that of Bender, but point out that an intermediate acetyl-imidazole is not obligatory, since in the case of N-methyl-imidazole, which is catalytically only slightly less efficient than free imidazole, the acylium ion could not be stabilized to form acyl-imidazole by loss of a proton, and so they suggest the alternative represented by reaction sequence II.



Reaction Sequence II

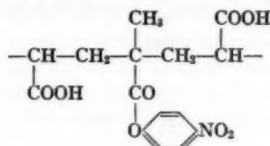
Brouwer *et al.* (190) have shown that in the later stages of the imidazole-catalyzed hydrolysis of acetic anhydride the rate of hydrolysis cannot be accounted for entirely by the rate of decomposition of N-acetyl-imidazole and suggest that like N-methyl-imidazole, N-acetyl-imidazole possesses catalytic power by virtue of the formation of diacetyl-imidazole. This intermediate would be very unstable, of the order of an acyl-pyridinium ion (193).

However, as Bender points out, even if the active site of chymotrypsin involves an imidazole ring, the mechanism is certainly more complex than a simple displacement by an imidazolyl side chain to give an acyl enzyme intermediate followed by hydrolytic cleavage, since in the catalytic hydrolysis, chymotrypsin is  $10^6$  times more efficient than imidazole. Also, the following differences are evident:

(a) Aliphatic alcohol esters are not split by imidazole but are by chymotrypsin. In this connection, Brouwer (190) has recently shown that imidazole

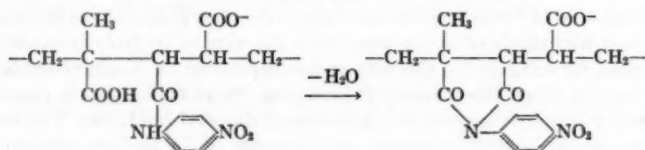
can catalyze the hydrolysis of dimethyl oxalate, but this is rather a special case of a very unstable ester. (b) The second imidazole in chymotrypsin, i.e., that not in the active center, is catalytically equivalent to free imidazole or imidazole in insulin. Thus, the primary imidazole must have a unique environment. (c) There is more selectivity in the deacylation step than would be expected from straight hydrolysis of acylimidazole [also, Brecher & Balls (191) find that, whereas in transesterification catalyzed by chymotrypsin there is a preference for long-chain alcohols, in the decomposition of  $\alpha$ -N-benzoyl-1- or 3-acetyl histidine methyl ester, there is no such preference].

To explain these large differences in efficiency of catalysis between model compounds and enzymes, the idea of "polyfunctional" or "intramolecular" catalysis (194), so elegantly developed by Swain & Brown (195), has received much attention in the last year. Bender (196) has studied the hydrolysis of phthalamic acid where the presence of a carboxyl group ortho to the amide bond increases its rate of hydrolysis by a factor of at least  $10^6$  to  $10^8$ , the reaction probably having phthalic anhydride as an intermediate. Zimmering *et al.* (197) have also studied the question by synthesis of a series of acrylic copolymers containing *p*-nitrophenyl and *p*-nitranilide groups in various orientations to carboxyl groups, and find an acceleration of ester hydrolysis by a factor of  $10^6$  at pH 5 in the copolymer illustrated (III).



III

They have also shown that an imidization reaction (IV):

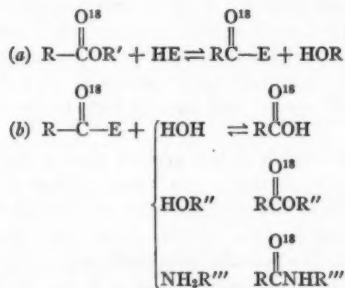


Imidization Reaction IV

has bell-shaped pH dependence, indicating that an unionized and an ionized carboxyl cooperate in the reaction. Herr & Koshland (198) have also seen a bell-shaped pH relationship in the acid hydrolysis of butyl thiophosphate; they propose a synchronous shifting of protons in a combined acid and base catalysis, and suggest that a similar mechanism may exist in enzyme catalysis.

## 2. KINETIC STUDIES, OBSERVATION, ISOLATION AND PROPERTIES OF INTERMEDIATES IN THE HYDROLYSIS OF ESTERS BY CHYMOTRYPSIN

*O<sup>18</sup> studies.*—Bender & Kemp (199) have shown that in the alkaline hydrolysis of ethyl- $\beta$ -phenylpropionate,  $O^{18}$ -labeled in the carbonyl oxygen, there is an exchange of  $O^{18}$  with its solvent water; however, in chymotryptic hydrolysis of this ester there is no  $O^{18}$  exchange, indicating that the mechanism is different in the two cases. When the same substrate was labeled with  $O^{18}$  in the alcohol moiety, there was rapid loss of  $O^{18}$ . In the presence of methanol,  $O^{18}$  was completely lost after 50 per cent hydrolysis, indicating that transesterification was occurring faster than hydrolysis. The  $O^{18}$  exchange from the carbonyl in alkaline hydrolysis can be explained in terms of the formation of a symmetrical intermediate during the hydrolysis, but in the case of chymotryptic hydrolysis, such an intermediate cannot be formed; the evidence was adduced as pointing to a double-displacement reaction (V):



Double-displacement Reaction V

In a second study (200), reaction (b) was demonstrated in the case of water and the rate constant for the exchange and  $K_m$  for the acid binding was found to be of the same order of magnitude as for hydrolysis of the corresponding amides (119, 201). With benzoyl-L-phenylalanine and acetyl-L-tryptophan there was rapid exchange, but with benzoyl-D-phenylalanine and  $\beta$ -phenylpropionate there was no exchange. These observations confirm the previous ones of Doherty & Vaslow (202) and Vaslow (203, 204) with N-acetyl-3,5-dibromotyrosine.

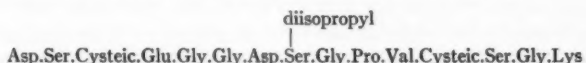
*Intermediates.*—The isolation of the acyl enzyme intermediate during the hydrolysis of *p*-nitrophenylacetate by chymotrypsin, previously reported by Balls & Aldrich (205) and Balls & Wood (206), has stimulated considerable study. Dixon & Neurath (136) developed methods by which the rates of the formation and decomposition of the acyl enzyme could be studied separately, and were able to show that:

(a) The same group which was acetylated by *p*-nitrophenylacetate could





observation that the aliphatic hydroxyl of a seryl residue bears the phosphoryl group. Schaffer *et al.* (211) have shown that a partial acid hydrolysis of *p*-chymotrypsin, so labelled, gives rise to the following peptides: Asp.Ser(PO<sub>4</sub>).Gly, Asp.Ser(PO<sub>4</sub>).Gly, Asp.Ser(PO<sub>4</sub>).Gly, Ser(PO<sub>4</sub>).Gly, indicating, in conjunction with previous observations, that Asp.Ser(PO<sub>4</sub>).Gly is a sequence common to the active centers of several esterases, including chymotrypsin, trypsin, and cholinesterase. The singularity of this observation is re-emphasized by the demonstration of Koshland & Erwin (212) that phosphoglucomutase, which binds phosphate in covalent linkage, during its catalytic transfer from glucose-1-phosphate to glucose-6-phosphate, also possesses an active center with a sequence Asp.Ser.Gly, with phosphate esterified onto the seryl. This observation was confirmed by Anderson & Jollès (213), who were able to isolate O-phosphoserine from P<sup>32</sup>-labelled phosphoglucomutase. Kennedy & Koshland (219) also showed that phosphoglucomutase was inhibited by DFP, but at concentrations much higher than with the esterases. Longer peptide sequences from P<sup>32</sup>-labelled phosphoglucomutase were obtained by a variety of degradative procedures, and the sequence Asp.Ser.Gly.Glu.Val was indicated, this being identical with that proposed by Schaffer *et al.* (211) for chymotrypsin. As a result, Koshland has suggested that phosphoglucomutase and other esterases possess the same basic bond-breaking mechanism, the common amino acid sequence being important in the activation of the seryl hydroxyl. Clearly, however, this basic bond-breaking mechanism is overlaid with other structural factors which determine the substrate specificity, and since in the case of phosphoglucomutase there is no hydrolysis, they also prevent a nucleophilic attack by water on the enzyme-bound phosphoryl. Dixon & Neurath (4) have reported the isolation of a number of large labelled peptides from a tryptic hydrolysate of diisopropylphosphoryl (P<sup>32</sup>)-labelled trypsin, oxidized with performic acid. The sequence of the smallest of these is (214):



and the peptide is derived from a large peptide containing 55 residues which possesses five cysteic acid residues and no histidine.

#### 4. SYNTHESIS OF EVIDENCE

Many workers have assumed that serine hydroxyl is the secondary point of attachment of phosphate, as the result of an N→O migration, the favorite mechanism being the initial formation of an unstable N-phosphoryl imidazole which subsequently donates the phosphoryl to serine. A major piece of evidence for this view has been the two-phase nature of the reactivation of organophosphorus-inhibited esterases by nucleophilic reagents, there being an initial reactivatable phase immediately after inhibition (215), passing into a non-reactivatable phase after several hours. The first stage is equated

with phosphoryl imidazole, and the second with phosphoryl serine. However, most of the evidence has been gathered from cholinesterase; diisopropylphosphoryl-chymotrypsin and trypsin have never been reactivated to a significant extent. Dixon & Neurath (216) showed previously that the attachment of the diisopropyl-phosphoryl group to trypsin was immediately to a stable linkage, with no unstable intermediate being detectable. Atkinson & Green (217) have synthesized N-diisopropylphosphoryl imidazole and studied its hydrolysis and reaction with hydroxamic acids, and have not found a close correspondence with the properties of inhibited cholinesterase. More powerful evidence has been provided recently by Ginjaar & Brouwer (218) who studied the proton exchange upon reaction of chymotrypsin with DFP; they observed the output of a single proton, whereas if a phosphoryl imidazole were formed, at least 1.7 protons would have been liberated. Kennedy & Koshland (219) have examined the stability of the phosphoryl bond of  $P^{32}$ -phosphoglucomutase and have found it to possess in the protein the properties of O-phosphoserine, while still being in equilibrium with substrate-bound phosphorus. The evidence thus excludes an N $\rightarrow$ O shift leading to the O-phosphoserine. Anderson & Jollès (213) have also shown that all histidine, tyrosine, and lysine residues are substituted by DNP in phosphophosphoglucomutase, as well as in the dephospho-enzyme.

On the other hand, Mounter *et al.* (220) have provided clear evidence to indicate that a basic group (pK 6.6 to 7.1) is involved both in the activity and DFP inhibition of chymotrypsin, trypsin, thrombin, cholinesterase, hog kidney DFP-ase (no inhibition), rabbit serum aliesterase, serum cholinesterase, and wheat germ lipase. In addition, as mentioned above, Dixon & Neurath (207) have shown a pK of 6.2 for the acetylation of chymotrypsin and trypsin, whereas Gutfreund & Sturtevant (221) found a pK of 6.7 for the acetylation of chymotrypsin by NPA. The spectral examination of monoacetyl-chymotrypsin (222) at pH 3 revealed, however, no detectable difference between the intermediate and chymotrypsin, indicating that in the stable acyl enzyme the acetyl group is not in the form of acetyl-imidazole. Gutfreund & Sturtevant (221) have also shown that there is a net uptake of 0.5 proton upon acetylation of chymotrypsin with *p*-nitrophenylacetate, which would preclude the acetylation of a basic group. They have postulated that this net uptake is due to the change in pK of an imidazole group from pK 6.4 to 7.3. Dixon & Neurath (223), however, have studied the spectrum of monoacetyl-chymotrypsin at alkaline pH, where rapid deacetylation was occurring, and were able to demonstrate the existence of an intermediate in the deacetylation with an absorption peak at 245 m $\mu$ , which corresponds closely with that described for acetyl-imidazole and the kinetics of whose decomposition also correspond to that of acetyl-imidazole.

Cunningham (224) has suggested a mechanism (Fig. 3) whereby many of these observations are correlated and become beautifully consistent:

The existence of a hydrogen bond between serine and a conjugate base

form of histidine, whereby the seryl oxygen is activated, accounts well for the pH dependence of activity, phosphorylation of DFP, and acetylation by *p*-nitrophenylacetate as well as for the immediate, stable binding of diisopropylphosphoryl or acetyl (at low pH). The formation of the new hydrogen bond between the acyl group and histidine also accounts for the increased  $pK$  observed in deacetylation. The observed dependence of the activity, DFP-inhibition and acetylation by *p*-nitrophenylacetate upon the struc-

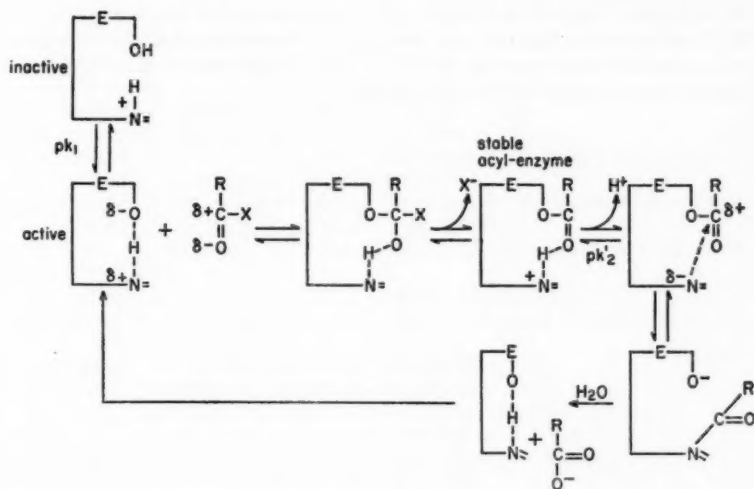


FIG. 3. Proposed mechanism of enzymatic hydrolysis.

tural integrity of the molecule is explained by the assumption that the histidine and serine are in different chains (or portions of chains), and would be separated by the disorientation in urea solution. The observation that a large peptide containing the serine but no histidine [Dixon & Neurath (214)] may be derived from trypsin lends weight to the assumption that the histidine and serine are well separated in the sequence, but not in space. The reactivity of the acetyl group toward hydroxylamine is also dependent on structural integrity and is lost reversibly upon urea denaturation, and would be explained by assuming that the acetylimidazolyl complex seen in hydrolytic deacetylation is also the reactive intermediate in reaction with hydroxylamine, and would not be formed when the imidazole is spatially separated from the acetylated seryl side chain.

Brouwer (225) has shown that in chymotrypsin one histidine is easily reactive to fluorodinitrobenzene under mild conditions, while one is sluggish; in

diisopropyl-pyosphoryl-chymotrypsin both are easily reactive, and he suggests that the breakage of a hydrogen bond between serine and imidazole occurs upon phosphorylation, thus making the imidazole nitrogen available for reaction. It might be observed that this bond probably could not re-form between the  $\text{—P=O}$  and histidine, as in the case of the acetyl enzyme, because of the steric hindrance of the alkyl groups on the phosphate. Jandorf *et al.* (226) have found conditions for the preferential dinitrophenylation of the sluggish histidine, and have shown that this inhibits activity and also the DFP reaction. They have interpreted this as evidence for direct reaction of DFP with histidine, but the only unequivocal observation that can be made is that histidine is involved in the DFP reaction, but whether directly or indirectly cannot be decided on this evidence.

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## BIOSYNTHESIS OF CHOLESTEROL AND RELATED SUBSTANCES<sup>1,2</sup>

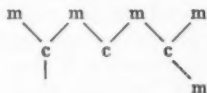
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In the reviews of lipid metabolism in the previous two volumes by Lynen (1) and by Kennedy (2) the recent dramatic advances in our knowledge of the oxidation and synthesis of fatty acids and their compounds have been very adequately reviewed. There has not appeared, however, in these volumes of recent years a critical appraisal of our knowledge concerning the biosynthesis of sterols and particularly of cholesterol. The last two years have witnessed important advances in this respect, almost as if the efforts of many years of previous work in various laboratories have suddenly come to fruition. Most of this review will be devoted therefore to the problem of the biosynthesis of the sterol structure as exemplified by the studies on cholesterol. The biosynthesis of steroid hormones and their relationship to cholesterol has been dealt with in great detail by Dorfman (3) and will not be discussed here. In order to assist the discussion of the present position of our knowledge, references to a few publications before 1956 will be made even though these may have been mentioned in some of the previous reviews. In this sense this article is not strictly an "annual" review except for the fact that it was mainly during the last year that the full meaning of many previous researches has become apparent. The evidence at hand will not necessarily be treated in a chronological order, but in a manner most suited for a reasoned presentation.

### BIOSYNTHESIS OF CHOLESTEROL FROM ACETATE

Perhaps the most fruitful idea concerning the mechanism of formation of cholesterol from acetate was the revival by Bloch (4) of the squalene hypothesis. It is to be recalled that when Würsch, Huang & Bloch (5) established that the acetate methyl (*m*) and carboxyl (*c*) carbons are arranged in the side-chain of cholesterol as shown in (I)



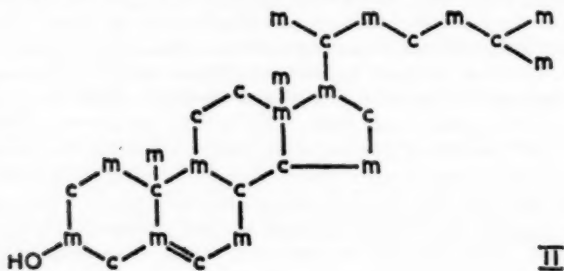
I.

<sup>1</sup> The survey of the literature pertaining to this review was completed in December 1957.

<sup>2</sup> The following abbreviations are used in this chapter: ATP for adenosine triphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); TPN for triphosphopyridine nucleotide; and TPNH for triphosphopyridine nucleotide (reduced form).

they were able to suggest a mechanism for the synthesis of cholesterol via isopentane (isoprenoid) units involving squalene as an intermediate. This hypothesis required among other things: (a) that squalene must be synthesized also from acetate by cells synthesizing sterols; (b) that squalene must be convertible into sterol; (c) that the distribution of acetate carbons in the sterol must be such as to be compatible with the distribution found in squalene, assuming a chemically and biologically feasible cyclization mechanism for the hydrocarbon, and (d) that a  $C_{30}$  sterol be found, containing all the 30 carbon atoms of squalene, and which was readily converted into cholesterol. All these criteria have been amply fulfilled.

The distribution of acetate carbons in the ring-structure of cholesterol synthesized by rat liver slices either from acetate-1- $C^{14}$  or acetate-2- $C^{14}$  has been the subject of systematic study by Cornforth and the writer and their colleagues. Special methods have been developed for the degradation of rings A and B [Cornforth, Hunter & Popják (6, 7)] and rings C and D [Cornforth, Popják & Gore (8); Cornforth, Gore & Popják (9)], which allowed the isola-

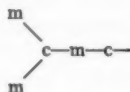


tion (with the exception of C-7) of all the carbon atoms of the sterol rings for isotope assay. Bloch (10) and Dauben & Takemura (11) reported on the origin of C-7. These investigations established the origin of all the carbons of cholesterol from either the methyl or carboxyl carbon of acetate (II). It was the invariable experience in these investigations that when acetate-1- $C^{14}$  was the precursor of cholesterol only one group of positions of the sterol became labelled. On the other hand, when acetate-2- $C^{14}$  was added to the liver slices, the cholesterol isolated contained one group of carbon atoms highly labelled and another group of lower isotope content (approximately one-tenth of the first group); the former group contained all the positions that did not derive isotope from acetate-1- $C^{14}$  and the latter those that became labelled from acetate-1- $C^{14}$ . The specific activities of the carbon atoms within each of the two groups were found equal to one another within experimental error. The fact that acetate-2- $C^{14}$  contributed some isotope to positions which acquired label from the carboxyl carbon of acetate could be explained readily by the formation of  $CH_3C^{14}OOH$  from  $C^{14}H_3COOH$  through the critic acid cycle. These results showed that every position in cholesterol had its origin in one

or the other of the carbons of acetate and that of the 27 carbons in cholesterol<sup>1</sup> 15 were derived from the methyl and 12 from the carboxyl carbon; this conclusion was reached by Little & Bloch (12) some time earlier on the basis of the ratio of  $C^{13}/C^{14}$  in cholesterol biosynthesized from  $C^{14}H_3C^{13}OOH$ .

The first step towards a knowledge of the intermediates involved in sterol biosynthesis was the demonstration by Langdon & Bloch (13) of the formation of  $C^{14}$ -squalene from  $C^{14}$ -acetate *in vivo* in the liver of the rat. They succeeded in isolating from the liver of rats, which were fed natural squalene and  $C^{14}$ -acetate, squalene containing significant amounts of isotope. Furthermore, when this  $C^{14}$ -squalene was fed to mice, in spite of its poor absorption, approximately 15 per cent of the administered dose was recovered as body cholesterol [Langdon & Bloch (14)]. Thus squalene appeared to be a much better precursor of sterol than any other substance tried previously. The synthesis of squalene from acetate was also demonstrated *in vitro* by liver slices and by various structures of the hen's ovary, which are also very active in respect to cholesterol synthesis [Popják (15)]. Schwenk, Todd & Fish (16) isolated  $C^{14}$ -squalene, without added carrier, from pig liver perfused with  $C^{14}$ -acetate. The conversion of  $C^{14}$ -squalene *in vivo* into cholesterol was confirmed in the writer's laboratory (Popják, unpublished), the average conversion in two groups of mice being 10 per cent of the administered dose.

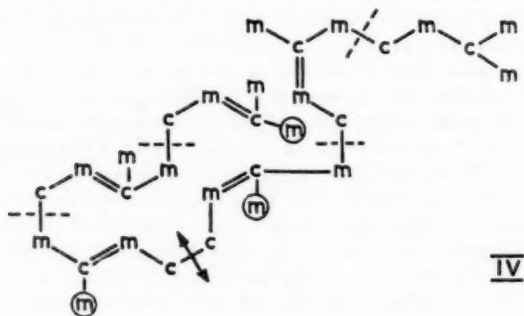
Bloch (4) suggested that if squalene were synthesized from acetate the acetate carbons might be arranged in the isoprenoid units of the triterpene as shown in III



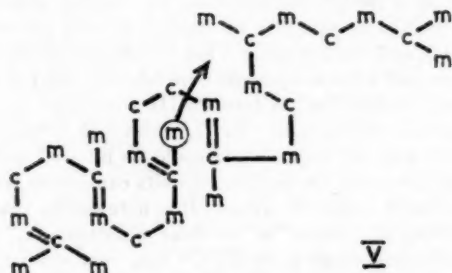
in analogy with the distribution suggested [Bonner & Arreguine (17)] in the isoprenoid units of rubber. Cornforth & Popják (18) prepared  $C^{14}$ -squalene synthesized by rat-liver lices from acetate-2- $C^{14}$  and carried out a carbon-by-carbon degradation of the labelled squalene. The method adopted was based on the initial cleavage of squalene by ozonolysis into acetone, laevulinic (4-keto-pentanoic), and succinic acids. They found that all the carbon atoms in all the six isoprenoid units of squalene were labelled, but the specific activity of the carbons marked "m" in formula III was about ten times higher than that of carbons corresponding to "c." Although  $C^{14}$ -squalene formed from acetate-1- $C^{14}$  was not degraded, it could be inferred quite justifiably that three of the carbons in the isopentene units came from the methyl and two from the carboxyl carbon of acetate. It is noteworthy that the ratio of the specific activities of carbons "m" to those of carbons "c,"  $m/c \approx 10$ , in squalene biosynthesized from acetate-2- $C^{14}$  was very similar to the ratio found in cholesterol formed from acetate similarly labelled [cf. Cornforth *et al.* (7, 9)].

Relating now the known distribution of acetate carbons in squalene to

the distribution predicted in cholesterol on the basis of variously proposed cyclization mechanisms of squalene [cf. Bloch (4); Woodward & Bloch (19)], the first pattern considered was as shown in IV. It was assumed that after



folding, formation of carbon-to-carbon bonds and elimination of three methyl groups marked with circles in IV, the  $C_{27}$  sterol structure could be formed. Comparison of this hypothetical arrangement of acetate carbons to that found experimentally in cholesterol (II) shows notable discrepancies, namely in the origin of carbons 7, 8, 12, and 13. The first discrepancy was noted by Woodward & Bloch (19), who isolated the two pairs of carbon atoms (C-18+C-13) and (C-19+C-10) from a sample of epiandrosterone prepared chemically from a sample of cholesterol biosynthesized from acetate- $2-C^{14}$ . Since at that time the origin of C-19 and C-10 was already known [cf. Cornforth *et al.* (7)] they were able to deduce that both C-18 and C-13 must have originated from the methyl carbon of acetate. This conclusion was confirmed later by Cornforth *et al.* (9) who isolated individually C-18 and C-13 from  $C^{14}$ -cholesterol by an independent method. Woodward & Bloch (19) consequently proposed another form for the folding of squalene (V), which



postulated the migration of a methyl group from either position 8 or 14 to position 13 and the subsequent elimination of three methyl groups in the



formation of cholesterol. The notable difference of this scheme, as compared to that shown in IV is that the line of symmetry of squalene falls between C-11 and C-12 and not between C-6 and C-7 of the sterol structure. The arrangement of acetate carbons in sterol demanded by this hypothesis turned out to be identical with that finally determined by experiment (cf. formula II).

The hypothesis proposed by Woodward & Bloch (19) had further implications of much interest. It suggested, firstly, that a trimethyl  $C_{30}$ -sterol may be an intermediate in the formation of cholesterol; the best candidate for this role being lanosterol, a 4:4:14-trimethyl sterol [Voser *et al.* (20)]. On the theoretical side, the "harmonica-like" folding of squalene, resulting in the proximity of double bonds, allowed a new stereochemical interpretation of the cyclization mechanism to be put forward and placed the ideas of a common biosynthetic origin for sterols and terpenes on to a more solid basis [Ruzicka (21); Eschenmoser *et al.* (22)]. The distinguished Swiss school of chemists led by Ruzicka expanded the classical isoprene rule into a "biogenetic isoprene rule" which may be applied now even to those terpenes whose carbon skeleton formerly could not in an obvious way be dissected into isoprenoid units. It is the remarkable feature of this hypothesis that, after certain well-defined assumptions are made, all the known  $C_{30}$  cyclic triterpenes and lanosterol may be derived with their full structural and configurational details from an all *trans*-squalene. It is assumed that all cyclizations of squalene are initiated by the attack of a cation (a hypothetical  $OH^+$ ) at one end of the squalene molecule folded in a specific way (e.g., chair-boat-chair-boat configuration for lanosterol) and once the reaction commenced it proceeds non-stop by electron shifts in a concerted manner, i.e. without the formation of stable intermediates. The cyclizing molecule of course carries a positive charge leading to the formation of an unstable carbonium ion which becomes ultimately stabilized by hydride and methyl shifts and not by hydration or proton elimination. The author expressed a few years ago the view in simpler terms that cyclic triterpenes and sterols have probably a common biosynthetic origin, the differences in their configuration being due to the steric specificity of the cyclizing enzymes [Popják (23)]. This, in terms of the hypothesis of the Swiss school, may be translated to mean the specificity of the type of folding of squalene and the processes involved in the stabilization of the intermediary carbonium ions. This "biogenetic isoprene rule" in relation to lanosterol is now supported to almost the last detail by experimental evidence as will be shown in further discussion.

#### STEROL INTERMEDIATES IN THE FORMATION OF CHOLESTEROL

It was Schwenk and his colleagues [cf. Schwenk, Todd & Fish (16)] who first observed that in various biological systems, synthesizing cholesterol from  $C^{14}$ -acetate, the cholesterol samples are contaminated with other  $3\beta$ -hydroxy sterols, the specific activities of which are higher than that of cholesterol. These contaminants have at first been called the "high counting

companions" of cholesterol whose presence in the sterol samples isolated at a short interval after the administration of  $C^{14}$ -acetate is particularly obvious. It was suggested that some of these sterols might be intermediates in the formation of cholesterol. Several sterols, lanosterol, 14-norlanostadienol, zymosterol (cholesta-8(9):24-dien-3 $\beta$ -ol) and desmosterol (cholesta-5:24-dien-3 $\beta$ -ol) have now been recognized as being among such high counting companions of cholesterol and involved in the lanosterol to cholesterol transformation.

In a number of papers Bloch and his colleagues adduced experimental evidence in support of the hypothesis that not only squalene but also lanosterol is an intermediate in the biosynthesis of cholesterol; in fact, the latter is a degradation product of the  $C_{30}$ -sterol. Clayton & Bloch (24) found that the unsaponifiable fraction of liver homogenates incubated with  $C^{14}$ -acetate contained in addition to  $C^{14}$ -cholesterol two radioactive  $C_{30}$ -sterols: lanosterol and agnosterol (4:4:14-trimethyl cholesta-7:9:24-trien-3 $\beta$ -ol). These were isolated by chromatography with added carrier "ischolesterol," which is a mixture of wool-fat sterols. When carrier "ischolesterol" was present throughout the incubations the yields of  $C^{14}$ -lanosterol and of  $C^{14}$ -agnosterol were greatly increased as compared to the controls containing no added "ischolesterol." The same stimulation could not be obtained by either pure lanosterol or agnosterol alone, but only by their combination. The other two main sterol constituents of "ischolesterol," dihydrolanosterol and dihydroagnosterol (both containing saturated side-chains) did not become labelled to a significant extent during incubations with liver homogenates and  $C^{14}$ -acetate. The occurrence and synthesis of lanosterol and of agnosterol in liver was not known formerly. Johnston, Gautschi & Bloch (25) also reported a method for the convenient preparation of lanosterol from "ischolesterol." The method depends on the initial separation of the 24:25-dibromo-derivative of lanosteryl acetate followed by debromination and hydrolysis of the acetate.

Schneider, Clayton & Bloch (26) have further demonstrated *in vivo* that both in the intestine and in the liver of the intact rat both squalene and lanosterol are synthesized from  $C^{14}$ -acetate. Ten minutes after the intraperitoneal injection of radioactive acetate, squalene, lanosterol, and a companion of lanosterol accounted for approximately one-third of the radioactivity in the nonsaponifiable fraction of intestine. Moreover, the specific activities of squalene and of lanosterol far exceeded at this time the specific activity of cholesterol. At a later interval, after the injection of  $C^{14}$ -acetate (75 min.), the bulk of radioactivity was present in cholesterol. In liver the accumulation of radioactive squalene and lanosterol was much less pronounced at the 10 min. interval than in the intestine, presumably because, on account of the more rapid sterol synthesis there, the peak values of these intermediates have already been passed. That lanosterol was indeed a precursor of cholesterol was shown by Clayton & Bloch (27) who incubated biosynthetic  $C^{14}$ -lanosterol with liver homogenates and found that 2.6 to 19 per cent of the added

lanosterol was recoverable as cholesterol. The higher percentage conversion was found when the amount of lanosterol introduced into the homogenates was reduced from 300  $\mu\text{g./ml.}$  of homogenate to 6.3  $\mu\text{g./ml.}$  When the percentage conversion to cholesterol was calculated on the basis of the amount of lanosterol disappearing (the amount of lanosterol "utilized") during the incubations, as much as 63 per cent of the lanosterol "utilized" could be accounted for as cholesterol. Olson, Lindberg & Bloch (28) studied in greater detail the enzymic conversion of lanosterol to cholesterol. They established that this is an oxidative process since the three "extra" methyl carbons of lanosterol are removed as carbon dioxide. No conversion of lanosterol to cholesterol could be observed anaerobically. These authors suggest that the 4:4-dimethyl and 14-methyl substituents of lanosterol are oxidized in steps to alcohol, aldehyde, and carboxyl groups and that they are eliminated by decarboxylation.

Tchen & Bloch (29) examined Ruzicka's hypothesis that the cyclization of squalene to lanosterol, for example, proceeds in a concerted manner. They prepared homogenates of liver such that could not carry the process of sterol biosynthesis beyond the stage of lanosterol and which could convert added squalene into lanosterol (see under Enzymes of Sterol Biosynthesis). They incubated  $\text{C}^{14}$ -squalene with liver homogenates in the presence of large excess of  $\text{D}_2\text{O}$  or  $\text{H}_2\text{O}^{18}$  in the incubation medium or with  $\text{O}^{18}$ -enriched oxygen as the gas phase. They found no excess deuterium or  $\text{O}^{18}$  in the  $\text{C}^{14}$ -lanosterol formed when labelled water was used in the incubations. On the other hand, when lanosterol was formed in the presence of  $\text{O}^{18}$ -gas, it contained considerable excess of  $\text{O}^{18}$  showing that the oxygen atom in lanosterol was derived from molecular oxygen. Tchen & Bloch (29) argue that the cyclization of squalene could not have been initiated by removal of a hydride ion followed by hydration as there was no incorporation of either deuterium or  $\text{O}^{18}$  into lanosterol from the water. They dismiss the idea that the cyclization may be proton-initiated (followed by stereospecific hydration) because in that event one might expect cyclization to take place even in the absence of oxygen and the product in this event to be lanosta-8:24-diene. They found no evidence of the formation of a cyclic hydrocarbon from squalene when oxygen was excluded from the incubations. Thus it seems that the cyclization process must be initiated by activated molecular oxygen and then proceeds to lanosterol without interruption or formation of stable intermediates. A detailed consideration of the mechanism of the cyclizing process is out of place here; at any rate, it must await further evidence on the nature of the activated form of oxygen and the determination of the type of methyl shift that occurs during cyclization (i.e., 1:3-shift involving the migration of the methyl group from position 8 to 13, or 1:2-1:2-methyl shift in which double migration from position 14 to 13 and from position 8 to 14 takes place). In view of the absolute specificity of the liver enzyme system to cyclize squalene to the lanosterol type of molecule, and in view of the oxidative nature of the reaction, Tchen & Bloch (29) proposed the term "squalene-

oxidocyclase I" for the liver enzyme. It is probable that there exist in nature a number of other squalene cyclizing enzymes specific for the variety of cyclic triterpenes.

The conversion of lanosterol into cholesterol raises the question whether intermediates are formed in the process or not. Schneider *et al.* (26) found in the nonsaponifiable fraction of liver and intestinal lipides of rats injected with C<sup>14</sup>-acetate a C<sup>14</sup>-sterol, distinct from lanosterol and cholesterol, which on the basis of its chromatographic behavior the authors thought to be a partial demethylation product of lanosterol. Gautschi & Bloch (30) examined this substance in more detail. It can be well separated from lanosterol by chromatography of the acetylated mixture of sterols (previously freed from cholesterol) on alumina, where it behaves as a substance somewhat more polar than lanosteryl acetate. Although less than 1 mg. of this substance was isolated, these authors adduced evidence by ingenious means that this new substance contains a double bond in the side-chain ( $\Delta^{24}$ ) and a nuclear double bond at an undetermined position ( $?\Delta^{8,9}$ ), that it contains a *gem*-dimethyl substituent at position 4, but that it contains no methyl substituent at C-14. When this substance derived from acetate-2-C<sup>14</sup> was incubated with liver homogenates capable of converting lanosterol into cholesterol, it gave radioactive cholesterol and two moles of CO<sub>2</sub> per mole of cholesterol formed. The evidence indicates, therefore, that this intermediate is a 4:4-dimethyl cholestadienol and that the conversion of lanosterol to cholesterol occurs in stages, the methyl group at position 14 being the first to be eliminated. Wells & Neiderheiser (31) reported the isolation of a new sterol from the feces of rats; it is suggested that this substance is 4 $\alpha$ -methylcholest-7-en-3 $\beta$ -ol, which might arise in the course of conversion of lanosterol to cholesterol. However, Djerassi, Mills, & Vilotti (32) isolated from the cactus *Lophocereus schottii* a new sterol, named lophenol, which was conclusively proved to be 4 $\alpha$ -methylcholest-7-en-3 $\beta$ -ol. The physical properties of lophenol differ so markedly from those of the substance isolated from the feces that their identity seems most unlikely. The occurrence in nature of the monomethyl substituted cholesterol adds further support to the view that the elimination of the three methyl groups from lanosterol occurs in stages.

The final stage in the biogenesis of cholesterol may be assumed to be the elimination of the 4-*gem*-dimethyl substituent, with the possible formation of a 4-monomethyl intermediate, the reduction of the double bond in the side-chain and the shift of the nuclear double bond to the 5:6 position. There is no definite evidence available at present to indicate in what sequence these reactions occur. The ease with which zymosterol (cholesta-8(9):24-dien-3 $\beta$ -ol) is converted into cholesterol both *in vivo* [Schwenk *et al.* (33)] and *in vitro* [Johnston & Bloch (34)] suggests that this substance is among the nearest precursors to cholesterol. Dihydrozymosterol (cholest-8(9)-en-3 $\beta$ -ol) is also converted to cholesterol in Bucher type of homogenates, but not by liver preparations made in a Waring blender, whereas zymosterol yields cholesterol in both types of enzyme preparations [Johnston & Bloch (34)]. This observa-

tion suggests that dihydrozymosterol is not an obligatory intermediate in the formation of cholesterol. Stokes, Fish & Hickey (35) have isolated from 11- to 14-days-old chick embryos injected with  $C^{14}$ -acetate two digitonide-forming sterols, which by weight represented about 2.3 per cent of the total sterols but contained as much as 26 per cent of the  $C^{14}$  incorporated into sterols. One of these substances was claimed to be cholesta-5:24-dien-3 $\beta$ -ol and was named desmosterol. The second substance was thought to be an isomer of desmosterol. Stokes *et al.* (35) quoted preliminary experiments which indicated conversion of desmosterol into cholesterol. The difficulties in deciding which of these transformations is an obligatory step in the formation of cholesterol have been fully discussed by Johnston & Bloch (34).

#### THE BIOGENESIS OF ERGOSTEROL AND SOME OTHER ISOPRENOID SUBSTANCES

There is increasing evidence to show that the mechanism of polyisoprenoid synthesis as revealed by studies on cholesterol is applicable to natural products derived from sources other than animal cells. Clearly, the results of Sonderhoff & Thomas (36) twenty years ago with deuterioacetate were the first to indicate the importance of acetate in the synthesis of ergosterol by yeast. Ottke *et al.* (37), by the use of an acetate-requiring mutant of *Neurospora crassa* and  $C^{14}H_2C^{13}O_2H$ , obtained results showing that at least 26 out of the 28 carbons of ergosterol must have been derived from acetate. That the general mechanism of ergosterol synthesis is similar to that of cholesterol is indicated by several lines of evidence. Hanahan & Wakil (38) found by chemical degradation of  $C^{14}$ -ergosterol, extracted from yeast grown in a medium containing acetate-1- $C^{14}$ , that C-23 and C-25 in the side-chain were labelled but C-24, C-26, C-27 and C-28 were not. Disregarding C-28, the labelling in the side-chain is thus similar to that found in cholesterol biosynthesized from acetate-1- $C^{14}$  (5). Dauben & Hutton (39) have shown that C-11 and C-12 in ergosterol are derived from the carboxyl carbon of acetate as in cholesterol (9). It was pointed out elsewhere in this review that C-11 and C-12 of cholesterol correspond, according to the Woodward-Bloch hypothesis, to the two central carbon atoms of squalene. Thus the origin of ergosterol via squalene as an intermediate has become very probable. This idea is further supported by the fact that among the yeast sterols may be found, although in small amounts, lanosterol and zymosterol [Schwenk *et al.* (40)] and that yeast synthesizes squalene also (34, 41). It seems, therefore, that the side-chain of the yeast sterol must become modified either during or after the cyclization of squalene. Danielsson & Bloch (42) have obtained  $C^{14}$ -ergosterol from baker's yeast grown aerobically in a medium containing glucose and  $C^{14}$ -formate. After chemical degradation of the side-chain of ergosterol, according to Hanahan & Wakil (38), all the radioactivity of the molecules was found to be in C-28. The experiments of Alexander, Gold & Schwenk (43) and of Alexander & Schwenk (44) indicate that the C-28 of ergosterol is derived through a transmethylation reaction from methionine. Alexander *et al.* (43) incubated cell-free homogenates of yeast with

$\text{NaHC}^{14}\text{O}_2$ ,  $\text{C}^{14}$ -formaldehyde, propionate-1- $\text{C}^{14}$  or -2- $\text{C}^{14}$ , methyl- $\text{C}^{14}$ -methionine and found more  $\text{C}^{14}$  derived from the methionine in unsaponifiable lipides and digitonin precipitable sterols than from any of the other labelled substances. Chemical degradation of the  $\text{C}^{14}$ -ergosterol derived from methyl- $\text{C}^{14}$ -methionine showed that C-28 contained about thirty times more  $\text{C}^{14}$  than other positions of the sterol. Further experiments by Alexander & Schwenk (44) demonstrated the direct transfer of the methyl group from methionine to the side-chain of ergosterol to become the substituent on C-24. When doubly labelled methionine (methyl- $\text{C}^{14}$ :methyl- $\text{H}^3$ ) was incubated with yeast homogenates, the  $\text{H}^3/\text{C}^{14}$  ratios in ergosterol from two experiments were  $0.97 \pm 0.06$  and  $1.02 \pm 0.02$ ; in the methionine the ratio was  $1.12 \pm 0.06$ . Thus a new transmethylation reaction from sulfur to carbon has been established. Since formate is known to be a source of carbon for labile methyl groups, the results of Danielsson & Bloch (42) may be explained on the assumption that labile methyl groups were first formed from the  $\text{C}^{14}$ -formate. This is supported by the findings of Alexander & Schwenk (44) that unlabelled methionine depressed the incorporation of  $\text{C}^{14}$  from formate into ergosterol from 6 per cent to 0.3 per cent, whereas formate did not decrease the incorporation of  $\text{C}^{14}$  from methyl- $\text{C}^{14}$ -methionine (24.8 per cent). Addition of homocysteine to the incubations doubled the yields of  $\text{C}^{14}$ -ergosterol from  $\text{C}^{14}$ -formate, and folic acid caused a further increase. Aminopterin, on the other hand, depressed the incorporation of formate. Formate plus homocysteine cut down sharply the incorporation of  $\text{C}^{14}$  from methyl- $\text{C}^{14}$ -methionine. Alexander, Gold & Schwenk (43) commented briefly that their experiments further indicated that squalene, but not zymosterol, is converted to ergosterol in yeast homogenates. Thus it seems possible that the methylation of the side-chain occurs immediately after cyclization of squalene to lanosterol.

The "biogenetic isoprene rule" of the Swiss school at first sight is not applicable to the  $\text{C}_{31}$ -triterpenes, as it offers no explanation for the origin of the one additional carbon. The work of Dauben and his colleagues on the biosynthesis of eburicoic acid is therefore of great significance, as it shows that 30 of the carbon atoms of this  $\text{C}_{31}$ -triterpene must have originated from squalene and that the additional carbon atom on the side-chain is derived from formate, probably through transmethylation from methionine as in the case of ergosterol. Dauben & Richards (45) grew the mould *Polyporus sulfureus* in a medium containing acetate-1- $\text{C}^{14}$  and have shown by chemical degradation of the isolated  $\text{C}^{14}$ -eburicoic acid that C-4, C-11, and C-12 were derived from the carboxyl carbon of acetate and that the specific activity of these carbons was such as to indicate that the whole molecule contained 12 labelled positions. These results are identical with those obtained with cholesterol. It was also shown that the carbons of the 4-*gem*-dimethyl groups in eburicoic acid and the C-21 carboxyl carbon are derived from the methyl-carbon of acetate, but that the methylene carbon (C-28) attached to the side-chain at C-24 does not come from either of the acetate carbons [Dauben,



Ban & Richards (46)]. When *P. sulfureus* was grown in the presence of  $C^{14}$ -formate,  $C^{14}$ -eburicoic acid was formed; it was shown by chemical degradation that 60 per cent of the radioactivity in the molecule was confined to the C-28 methylene carbon which had a specific activity 48 times greater than the other carbons in the molecule and which were equally labelled [Dauben, Fonken & Boswell (47)]. These results are then analogous to those obtained with ergosterol and extend the "biogenetic isoprene rule" to the  $C_{28}$ -triterpenes.

Among various other isoprenoid substances whose biosynthesis has been studied in recent years is  $\beta$ -carotene and lycopene. It was shown some years ago by Grob and his colleagues (48) that acetate was utilized for the synthesis of  $\beta$ -carotene by *Phycomyces blakesleeana*. Partial degradation of  $\beta$ -carotene- $C^{14}$  synthesized from labelled acetate showed that the distribution of acetate carbons in the isoprenoid units was similar to that found in squalene [Grob & Büttler (49, 50)]. The similarity of the mechanism of biosynthesis of  $\beta$ -carotene and squalene is emphasized further by the results of Braithwaite & Goodwin (51) who showed that mevalonic acid acts as the source of the isoprenoid units in  $\beta$ -carotene synthesis just as it does for squalene (cf. next section). In view of all this, the results of Zabin (52) on lycopene, a typical carotenoid, are most surprising, as they do not fit any of the schemes of polyisoprenoid synthesis. This investigator injected picked ripening tomatoes with either acetate-1- $C^{14}$  or acetate-2- $C^{14}$  and then allowed the ripening process to continue for 5 to 12 days.  $C^{14}$ -lycopene was isolated and degraded by ozonolysis into acetone, laevulinic, acetic and formic acids. The specific activity of the total carbon of laevulinic acid was the same as that of the lycopene itself and, since the laevulinic acid contained all five types of carbon atoms [ $-w-x(w')-y-z-$ ; cf. Klyne (53)] of the isoprenoid units of lycopene, it could be inferred that this carotenoid is formed by the condensation of identical units. On the other hand, the distribution of  $C^{14}$  among the carbons of the isoprenoid units as deduced from the radioactivity of acetone (carbons  $w-x-w'$ ), acetic acid (carbons  $w'-x$ ) and formic acid (mixture of carbons  $y, z$  and  $w$ ) allows no interpretation at present of the possible way in which the isoprenoid units are built up in the fruit from acetate. Indeed, so strange are the results that a re-examination of the question is warranted. The writer thinks that the carbon-by-carbon degradation of laevulinic acid (18), which was not carried out, might have given more reliable results than the deductions based on the radioactivities of acetic and formic acids, the products of the ozonolysis that may have arisen from positions other than predicted by theory. On the other hand, it is possible that during the relatively long period (5 to 12 days) of ripening, extensive randomization of acetate carbons took place with incorporations into various constituents of the tomatoes so that the process of isoprenoid synthesis has become obscured. It is, of course, equally possible, as Zabin (52) suggests, that the mechanism of formation of lycopene has no relation to the mechanism of formation of isoprenoid compounds in animal tissues or that the reactions leading to the



formation of the polymerizing units are different in different organisms, although in view of the wide experience now with animal tissues, moulds, yeast, and plants this is most unlikely.

#### MEVALONIC ACID AND OTHER BRANCHED-CHAIN ACIDS IN SQUALENE AND CHOLESTEROL SYNTHESIS

The discovery of mevalonic acid (or lactone) (3-hydroxy-3-methylpentano-5-lactone) and of its participation in sterol synthesis was recorded briefly in the review by Kennedy (2). Since the discovery by Tavormina, Gibbs & Huff (54) that mevalonic acid as a precursor of cholesterol is far superior to any other substance previously tried, several reports appeared which threw more light on the role of this new substance in sterol biosynthesis. Methods for the synthesis of  $C^{14}$ -labelled mevalonic acid have been reported by Hoffman *et al.* (55) and independently by Cornforth *et al.* (56, 57) and by Eggerer *et al.* (58).

Since the results of Tavormina *et al.* (54) and of Tavormina & Gibbs (59) suggested that mevalonic acid may donate directly five of its carbon atoms as isoprenoid units for sterol synthesis, Cornforth *et al.* (56, 57) have examined the question in detail. They found that mevalonic acid-2- $C^{14}$  was also an efficient precursor of squalene; rat liver homogenates which formed  $C^{14}$ -cholesterol from mevalonic acid-2- $C^{14}$ , formed under anaerobic conditions  $C^{14}$ -squalene exclusively. When the  $C^{14}$ -squalene was degraded chemically, it was found to contain only six labelled positions: one in each of the terminal isopropyl groups and four within the chain of the molecule; the branched methyl groups were not labelled. The results thus indicated that C-2 and the  $\beta$ -methyl carbon of mevalonic acid retained their individuality during biosynthesis and that an asymmetrically labelled isoprenoid unit was derived from mevalonic acid. The synthesis of squalene from mevalonic acid therefore must proceed by the coupling of C-2 of one molecule to the C-5 of another. Similar results were reported by Dituri, Gurin & Rabinowitz (60), although their method of degradation did not give as clear-cut an answer as the work just quoted above.

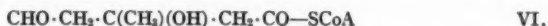
As all experimental evidence indicates that the conversion of squalene to cholesterol, through lanosterol as an intermediate, occurs according to the general scheme put forward by Woodward & Bloch (19), it was possible to predict (56, 57) that cholesterol biosynthesized from mevalonic acid 2- $C^{14}$  would contain only five labelled positions: C-1, C-7, C-15, C-22 and either C-26 or C-27; in lanosterol a sixth position, one of the two methyl carbons attached to C-4, must be also labelled. Confirmation of this pattern of labelling came from Isler *et al.* (61) who reported the partial degradation of  $C^{14}$ -cholesterol biosynthesized from mevalonic acid-2- $C^{14}$ ; they found that C-7, C-22 and either C-26 or C-27 were labelled. The results further indicated that the cholesterol must have contained only five labelled positions and that carbon atoms other than C-22 and C-26 (or C-27) in the side-chain were not labelled.

Mevalonic acid is utilized also *in vivo* for cholesterol synthesis. Gould & Popják (62) found that within 1 hr. after intraperitoneal injection into mice approximately 40 per cent of the administered dose was recoverable in liver and carcass cholesterol and 10 per cent in respiratory carbon dioxide. Very nearly one-half of the injected dose was excreted in 4 hr. in the urine of rats. It is possible that the substance excreted in the urine was the biologically inactive enantiomorph of the synthetic DL-acid, although this has not been ascertained yet. It is noteworthy that the amount of  $C^{14}O_2$  expired by the mice was only a little more than may be expected on the basis of the conversion of lanosterol into cholesterol, i.e. by the loss of one labelled position (4-methyl) from lanosterol. In the *in vivo* experiments quoted above and in several experiments *in vitro* carried out in the writer's laboratory with liver homogenates the fatty acids acquired only very feeble labelling from 2- $C^{14}$ -mevalonic acid, which does not appear to be degraded to acetate. In liver enzyme preparations containing the soluble fraction of homogenates and microsomes, which actively synthesize cholesterol from mevalonic acid (or acetate), acetyl-coenzyme A is readily formed from endogenous or from added substrates. When acetoxyhydroxamate (presumably derived from acetyl-coenzyme A) was isolated by chromatography from such preparations after incubation with mevalonic acid-2- $C^{14}$  or -1- $C^{14}$  no trace of radioactivity was found in the hydroxamate.

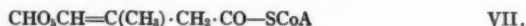
All evidence indicates therefore that mevalonic acid is the direct source of the isoprenoid units in squalene and sterol biosynthesis, although the chemical nature of condensing units has not been determined yet. It is also a matter of conjecture at present when the loss of C-1 of mevalonic acid occurs; a knowledge whether the carboxyl-carbon is lost before or after, or synchronously with the condensation of the mevalonic units would help greatly in establishing the nature of the reactions of squalene synthesis.

Amdur, Rilling & Bloch (41) made use of a yeast enzyme system (see under Enzymes of Sterol Synthesis) synthesizing squalene from mevalonic acid to study some questions of the mechanism of the synthesis. When 2- $C^{14}$ :5-tritio-mevalonic acid was employed as substrate, they found that the ratio of  $C^{14}/H^3$  in the squalene formed was the same as that in the mevalonic acid added to the incubations. This observation indicates that the alcohol group in position 5 of mevalonic acid is not oxidized either to aldehyde or carboxyl during the biosynthetic reactions. It would seem therefore that 3-hydroxy-3-methyl glutaraldehydic acid, synthesized and named mevaldic acid by Shunk *et al.* (63), is not on the pathway from mevalonic acid to squalene, but is perhaps the precursor of mevalonic acid. It has been reported (63) that mevaldic acid inhibited the incorporation of  $C^{14}$  into cholesterol from acetate-1- $C^{14}$  in rat liver homogenates to about the same degree as unlabelled mevalonic acid. In microbiological assays carried out on *Lactobacillus acidophilus* (ATCC 4963), mevaldic acid had an activity only 1/200th of that of mevalonic acid. Eggerer *et al.* (58) reported also the chemical synthesis of mevaldic acid by methods virtually identical with those of

Shunk *et al.* (63), i.e., by the Reformatsky reaction between the dimethyl-acetal of acetoacetaldehyde and ethyl bromoacetate. Eggerer *et al.* (58) and Lynen (64) consider that on chemical grounds mevaldic acid, or its coenzyme A derivative (VI),



may fulfil particularly well the role of the biological source of isoprenoid units, for after dehydration the CoA derivative of 3-methyl glutaconaldehydic acid (VII)



would be particularly active to form polyisoprenoid structures by virtue of its aldehyde and reactive methylene-group. Mevaldic acid is an unstable compound and the ready formation from it of 3-methylcrotonaldehyde has been recorded by Shunk *et al.* (63) and by Lynen and his colleagues (58) also. Although Lynen's ideas are very attractive, further experimental evidence supports the view that mevaldic acid may be the precursor of mevalonic acid rather than derived from it. Wright *et al.* (65) found that in liver homogenates in which the utilization of mevalonic acid for cholesterol synthesis has been blocked by preliminary incubation of the homogenates by ribonuclease [Wright & Cleland (66)], mevalonic acid was formed from mevaldic acid.

Tchen (67) reported the formation of a new substance from mevalonic acid by a yeast enzyme fraction. The substance still contains the carboxyl carbon. It is claimed that it is a phosphorylated derivative (either the 3- or 5-phosphate) and that it is converted to squalene by the yeast enzyme system described by Amdur *et al.* (41). A substance formed from mevalonic acid in liver enzyme preparations and with properties not unlike those described by Tchen (67) has been isolated in the writer's laboratory by chromatography on paper and on Dowex-2 (formate form), but the chemical identity of this substance is as yet undetermined; it is converted by liver preparations into sterol nearly twice as efficiently as DL-mevalonic acid (Popják & de Waard, unpublished).

Cornforth *et al.* (56, 57) considered the various possible transformations mevalonic acid (or lactone) might undergo before the formation of squalene. As dehydration seemed a likely event, they synthesized all the anhydro compounds derivable from mevalonic acid (except *trans*-5-hydroxy-3-methylpent-2-enoic acid) labelled with  $\text{C}^{14}$  in position 2; the substances made were: (a) 3-methylpent-2-eno-5-lactone and (b) the *cis*-acid derived from it; (c) *cis*-3-methylpenta-2:4-dienoic acid; (d) 3-hydroxy-3-methylpent-4-enoic acid; (e) 3-methylpenta-2:4-dienoic acid (mixture of *cis* and *trans* acids); and (f) 5-hydroxy-3-methylpent-3-enoic acid (mixture of *cis* and *trans* acids). None of these labelled compounds gave significant labelling in cholesterol when incubated with liver homogenates. The wholly negative results may mean that not one of these substances is on the path of the conversion of

mevalonic acid to squalene and cholesterol or that an enzyme required to make an activated form of such intermediate(s) is lacking. It is conceivable that mevalonic acid, by dehydration and decarboxylation, may give rise to isoprene itself, which could readily polymerize by electron shifts induced by the attack of a cation. This idea was tested by Cornforth *et al.* (57) in an experiment in which a liver preparation was incubated for 2 hr. in a sealed system with mevalonic acid-2- $C^{14}$  and unlabelled isoprene. At the end of the incubation the isoprene was trapped into a receiver cooled with liquid air and containing a saturated solution of sulfur dioxide. The isoprene- $SO_2$  polymer which was isolated contained no traces of radioactivity, although the isoprene (most of which existed of course in the vapor phase at the temperature of the incubation, 37°) depressed the incorporation of mevalonic acid into sterol to one-half of the control value. It is extremely doubtful if the isoprene diluted the radioactivity derived from mevalonic acid; it is more probable that isoprene was toxic to the preparation. In a private communication, Dr. K. Bloch informed the writer that his attempts to demonstrate conversion of isotopically labelled isoprene into squalene have so far been unsuccessful.

The experience of Cornforth *et al.* (57) with isoprene raises a general problem arising out of many experiments published in recent years and in which isotopically labelled precursors have been used in the study of various biosynthetic processes. It is often desired to establish the nature of an intermediate derived from a labelled precursor and for this purpose unlabelled substances suspected of being intermediates are added to the incubations in an attempt to "dilute" the label in the product derived from the labelled precursor. The writer thinks that observed "dilutions" should be treated with greater reserve of judgment than has been done in the past. The negative results of Cornforth *et al.* (56, 57) with the "anhydro" derivatives of mevalonic acid have already been discussed, but it may be worth recording here that 3-methyl-pent-2-eno-5-lactone and 5-hydroxy-3-methyl-pent-2-enoic acid both depressed the incorporation of  $C^{14}$  from mevalonic acid-2- $C^{14}$  by about 70 per cent, although neither of the unsaturated compounds, when labelled with  $C^{14}$ , gave more than negligibly small labelling in cholesterol. We may have to consider, therefore, that the  $\alpha,\beta$ -unsaturated compounds (lactone and acid), by virtue of the similarity of their chemical structure to that of mevalonic acid, acted as competitive inhibitors. This question will be examined again in the light of some further publications.

Gey *et al.* (68) have examined various  $C_5$  and  $C_6$  branched chain acids (isovaleric, 3-methylcrotonic, 3-hydroxyisovaleric, *cis*- and *trans*-3-methylglutaconic and 3-hydroxy-3-methylglutaric acid), suspected during the past years as being possibly connected with the origin of isoprenoid units in sterol and terpene biosynthesis, for their ability to suppress incorporation of  $C^{14}$  into cholesterol from acetate-1- $C^{14}$  in liver homogenates. Mevalonic acid was also included among the substances tested. When these branched acids were added to the homogenates in amounts twenty times greater (on the basis of molar ratios) than acetate-1- $C^{14}$ , the  $C_5$  acids (isovalerate, methylcrotonate

and hydroxy-isovalerate) inhibited incorporation of  $C^{14}$  into cholesterol only slightly (approximately 15 to 20 per cent inhibitions); *cis*- and *trans*-methylglutaconate were without effect, hydroxymethylglutarate on the other hand caused a profound inhibition (80 to 90 per cent), this being of the same order of magnitude as caused by mevalonic acid. The authors conclude that the probable meaning of these results is that hydroxymethylglutarate, formed from acetate, is a precursor of mevalonic acid and hence of cholesterol and that the  $C_5$  acids are not on the main pathway of sterol biosynthesis, but are by-products of metabolism of hydroxymethylglutarate. Such conclusion regarding the role of this acid is not entirely compatible with all the known experimental facts. If the strong inhibition of acetate incorporation into cholesterol caused by hydroxymethylglutarate were due to its being readily converted into mevalonic acid, then one would expect isotopically labelled hydroxymethylglutarate to be nearly as good a carbon source for cholesterol synthesis as mevalonic acid. The results of Tavormina, Gibbs & Huff (54) showed that this was not the case; in comparison with mevalonic acid  $Me-C^{14}$ -hydroxymethylglutarate donated only minute amounts of  $C^{14}$  to cholesterol in liver homogenates. There are two further explanations worth considering: (a) that hydroxymethylglutarate by a rapid cleavage to acetyl-CoA and acetoacetate [cf. Rudney (69)] may have diluted the radioactivity of the acetate; and (b) that it acted largely as a metabolic inhibitor rather than as a precursor in cholesterol biosynthesis. On the basis of the experimental details provided (volumes of homogenates and concentrations of substrates added) explanation (a) is on the borderline of possibility. In view of some results of Wright (70) to be discussed later, hydroxymethylglutarate in amounts used by Gey *et al.* (68) may turn out to be an antimetabolite of sterol and terpenoid biosynthesis.

Gey *et al.* (68) examined, in addition to the substances listed above (a) 3-methylbutan-1-ol (isoamylalcohol), (b) 3-methylbut-2-en-1-ol, (c) 3-methylbutan-1:3-diol, (d) 3-methylcrotonaldehyde, (e) 3-methyl-3-hydroxybutyraldehyde, (f) 3-methylbut-1-en-3-ol, (g) 3-hydroxy-3-methylpent-4-enoic acid and (h) 3-methylpent-3-enoic acid for their ability to inhibit incorporation of acetate carbon into cholesterol. Compounds (c) and (e) were without effect, the other substances caused inhibitions ranging from about 20 to 60 per cent. It is worth mentioning that 3-hydroxy-3-methylpent-4-enoic-acid-2- $C^{14}$  tested by Cornforth *et al.* (57) gave no  $C^{14}$ -cholesterol when added to liver homogenates.

When all the evidence published during the last five years on the possible participation of the various  $C_5$  and  $C_6$  branched chain acids, excepting mevalonic acid, in the synthesis of polyisoprenoid substances, and particularly of sterols, is sifted down, there is only one, which taken at its face value, might suggest the direct derivation of an isopentane (or isopentene) structure from some of the  $C_5$  or  $C_6$  acids. Bloch, Clark & Harary (71) compared the utilization of 3-hydroxy-3-methylglutaric, of *cis*-3-methylglutaconic, of 3-hydroxyisovaleric and of 3-methylcrotonic acids (all labelled on C-3 with

C<sup>14</sup>) for the synthesis of cholesterol *in vivo* and *in vitro* with liver homogenates. In comparison with acetate all four branched-chain acids were very poor sources of carbon for cholesterol synthesis in homogenates, but *in vivo* 3-C<sup>14</sup> methylcrotonate exceeded the utilization of acetate by a factor of 2-3. 3-C<sup>14</sup>-hydroxymethylglutarate and 3-C<sup>14</sup>-hydroxyisovalerate were only one-half to one-third as efficient as acetate as sterol precursors *in vivo*. When partial degradations of samples of C<sup>14</sup>-cholesterol, obtained from animals given 3-C<sup>14</sup>-methylcrotonate, 3-C<sup>14</sup>-hydroxymethylglutarate and 3-C<sup>14</sup>-hydroxyisovalerate were carried out the results suggested that the cholesterol samples derived from each substrate contained only six labelled positions, and not 12 as might be expected if the three acids had been broken down first to acetate giving rise to acetate-1-C<sup>14</sup>. The six positions that might be expected to be labelled in cholesterol from the 3-C<sup>14</sup>-branched-chain acids, if breakdown to acetate or other more complex interconversions do not occur, are 4, 10, 8, 14, 20 and 25. Unfortunately, not one of these crucial carbon atoms was actually isolated by itself; the specific activities of C-25 and of C-10 were calculated. The authors were careful to point out that while their results suggested that the carbon chains of the three branched-chain acids were used for cholesterol synthesis without breakdown to acetate, the utilization of the two hydroxy-acids was not of the order of magnitude that would be expected from specific precursors. The results with methylcrotonate were regarded in a more favourable light. However, in an elegant essay, devoted to the problem of the biosynthesis of branched-chain compounds and their relationship to polyisoprenoid and sterol synthesis, Bloch (72) points out that if their former assumption that the intact molecule of 3-methylcrotonic acid entered the condensation reaction in sterol biosynthesis was correct, then the efficiency of conversion "should be independent of the location of the carbon label in the precursor". He reports that this was not true for methylcrotonate since 1-C<sup>14</sup>-methylcrotonate was less efficient as a precursor of cholesterol than the compound labelled in position 3. Furthermore C<sup>14</sup> from 1-C<sup>14</sup>-methylcrotonate was found at C-25 of cholesterol in amounts indicating its extensive breakdown to acetate-1-C<sup>14</sup>. Thus a conflicting picture emerged: the branched-chain acid appeared to be a direct cholesterol precursor only when it was labelled in the isopropyl group. This is not unlike the experience gained previously with isovalerate [Zabin & Bloch (73)]. A cyclic regeneration of 3-methyl crotonic acid via 3-hydroxyisovaleric, and 3-methylvinylacetic acid and fixation of CO<sub>2</sub> by the latter to give *trans*-3-methylglutaconic acid followed by decarboxylation of the latter at the carboxyl carbon originally derived from 3-methylcrotonic acid would result (a) in "preservation" of the label at C-3, (b) in preferential loss of label from C-1 and (c) equilibration of C-4 and C-3 with C-2 in methyl crotonic acid [Bloch (72)]. This is illustrated in Figure 1.

This picture is further complicated by the probable interconversion of 3-methylglutaconic with 3-hydroxy-3-methylglutaric acid or its coenzyme A ester. Kennedy (2) has reviewed the recent knowledge of the metabolism of



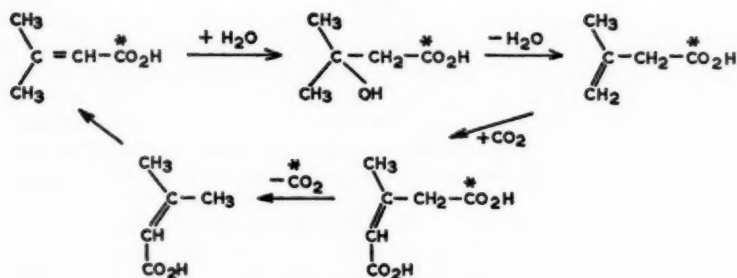
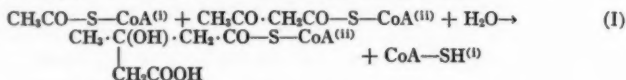


FIG. 1. Cyclic regeneration of 3-methylcrotonic acid (72).

these closely interrelated branched-chain acids. One further point came to light recently. Rudney (69) studied both the synthesis and cleavage of 3-hydroxy-3-methylglutarate. He finds that the reactants in the synthesis with a purified yeast enzyme were acetyl-CoA and acetoacetyl-CoA and that the reaction proceeded according to equation (I), its product being the mono-coenzyme A derivative of 3-hydroxy-3-methylglutaric acid.





exclude as an intermediate a substance whose specific activity is lower than that of the product, nevertheless all the evidence at hand now supports the authors' conclusions. The reviewer would like to mention that some years ago he suggested, among other possible explanations for the results, that squalene may not be an obligatory intermediate in cholesterol biosynthesis (15). This suggestion was made because the specific activity of squalene isolated from liver slices incubated with  $C^{14}$ -acetate was much too low to account for all the  $C^{14}$  incorporated into cholesterol. No doubt the real explanation of the observations is the lack of equilibration of the carrier squalene added to the slices with the metabolic squalene synthesized *de novo*, which is rapidly converted into cholesterol. Such an explanation probably cannot be applied to the observations of Adamson & Greenberg (75) whose conclusions are based on wider evidence.

Some suggestions as to the nature of terpenoid intermediates in the synthesis of squalene must be also discussed. Dituri *et al.* (77) found that  $C^{14}$ -squalene was formed from methyl- $C^{14}$ -3-hydroxy-3-methylglutarate in supernatants of liver homogenates centrifuged at 100,000 g for 30 min. Geraniol, linalool and geranic acid when added to the incubations had no effect on  $C^{14}$  incorporation into squalene from hydroxymethylglutarate. Farnesol and farnesinic acid, however, depressed very much the labelling of squalene. When the farnesol and farnesinic acids were isolated from the incubations with methyl- $C^{14}$ -hydroxymethylglutarate, the alcohol contained no  $C^{14}$  but the acid was significantly labelled. It was suggested that farnesinic acid is an intermediate in squalene synthesis. The biosynthesis of farnesinic acid in animal tissues is of very considerable interest; it is therefore a great pity and somewhat confusing that different lines of evidence seem to exclude farnesinic acid as intermediate in squalene biosynthesis. Wright & Cleland (66), referring to the work of Dituri *et al.* (77), quote Dr. S. Gurin as having stated at a conference in December, 1956, that hydroxymethylglutarate is no longer considered an intermediate in sterol biosynthesis and furthermore that it is believed that a  $C^{14}$ -labelled and biologically active impurity (?mevalonic acid) contained in the samples of methyl- $C^{14}$ -hydroxymethylglutarate used by Gurin and his colleagues was responsible for the results just discussed. The reviewer had not seen any other reference to this announcement by Gurin or writing by him, but it was thought to be of such importance as deserved the attention of readers.<sup>3</sup>

Sandermann & Stockmann (78) reported briefly that  $C^{14}$ -farnesinic acid synthesized by the Reformatsky reaction from geraniolacetone was not a precursor of cholesterol in rat liver. But perhaps the much stronger evidence against the idea of farnesinic acid being an intermediate in squalene synthesis comes from the observations of Amdur *et al.* (41), who found that the ratio of  $C^{14}/H^3$  in squalene biosynthesized from 2- $C^{13}$ :5- $H^3$ -mevalonic acid was the same as in the mevalonic acid itself. If farnesinic acid had been formed as an

<sup>3</sup> Since this review was sent to press, a paper by F. Dituri, J. L. Rabinowitz, R. P. Hullin & S. Gurin containing experimental evidence on this question appeared in the *J. Biol. Chem.*, 229, 826 (1957). Because of the delay in publication of this number of the journal, the article could not be included in the survey.

intermediate one would have expected the loss of tritium from two out of six molecules of mevalonic acid since the two central carbon atoms in squalene, which might be expected to be derived from the carboxyl carbon of two farnesinic acid molecules, originate from C-5 of mevalonate [Cornforth *et al.* (56, 57)]. The finding by Wright & Cleland (66) that farnesol and farnesinic acid caused 30 to 100 per cent inhibition of incorporation of  $C^{14}$  into cholesterol from 2- $C^{14}$ -mevalonate in liver homogenates coupled with the experiments of Wright (70), showing that both farnesol and farnesinic acid have a strong antimetabolite action on *Lactobacillus acidophilus* (ATCC 4963) in the presence of mevalonic acid, argue very strongly in favour of the contention that these two substances are metabolic inhibitors even in the liver. Wright (70) showed further that even hydroxymethylglutarate was a powerful inhibitor of the growth of *L. acidophilus* in the presence of mevalonate. The strong inhibitory action of hydroxymethylglutarate on incorporation of  $C^{14}$  from acetate into cholesterol (68) in liver preparations may therefore be also connected with a metabolic inhibition rather than with a precursor role of hydroxymethylglutarate.

The results with mevalonic acid that have been obtained so far stand out in striking contrast with the rather indecisive and often conflicting results that have been reported with other  $C_6$  or  $C_8$  branched-chain acids. The presumptive evidence is of course that mevalonic acid is the intermediate formed from acetate, but as pointed out elsewhere in this review, its formation must be one of the most severely rate-limiting reactions in the whole gamut of reactions of cholesterol synthesis. Mevalonic acid seems to be a most unique intermediate in metabolism in that once it is formed it is probably not used to a significant extent for purposes other than polyisoprenoid synthesis if one is to judge by its behaviour after injection into animals. It would be futile to offer any speculation as to the relationship between this and other branched-chain acids at present. Some may feel that a large amount of effort had been wasted in analysing the relationship between branched-chain acids, such as hydroxymethylglutarate and substances related to it, and sterol biosynthesis. To the respondents one would like to offer the thought that, if hydroxymethylglutarate had not been suggested as a source of isoprenoid structures [Bloch (4)], mevalonic acid, in spite of its discovery, may possibly not have been tested yet in this role.

#### ENZYMES OF STEROL SYNTHESIS

The cell-free homogenates of liver, introduced first by Bucher (79) and Frantz & Bucher (80) for the study of sterol biosynthesis have been used with success in various laboratories for some years now. The introduction of these preparations was perhaps one of the most important advances in the study of the biosynthesis of cholesterol since the demonstration by Bloch, Borek & Rittenberg (81) of the ability of liver slices to synthesize cholesterol from labelled precursors. A detailed examination of the components of these homogenates involved in the biosynthesis of cholesterol from acetate has

been reported by Bucher & McGarrah (82). The evidence presented clearly showed that the mitochondria were not involved in the reactions; microsomal fractions and the soluble protein part of the homogenates (supernatants after centrifuging at 105,000 g) together were required for the synthesis. Neither the microsomes nor the soluble fraction were able to carry out the synthesis alone, only their combination being effective. Microsomes sedimented between 5,000 and 30,000 g and between 30,000 and 60,000 g were more active than the particles sedimented between 60,000 and 105,000 g. Although the mitochondrial fraction (sediment obtained at 5,000 g) when combined with the soluble supernatant of homogenates provided a synthetic system, it seems very probable that the relatively feeble activity was attributable to the presence of microsomes among the mitochondria. The ability of the microsomal fractions to promote cholesterol synthesis was roughly parallel to their cholesterol content, but bore no relationship to their ribonucleic acid content. When the liver of rats injected intravenously with acetate-1-C<sup>14</sup> was homogenized 3 or 30 minutes after the injection of the animals, it was found that 84 to 91 per cent of the total C<sup>14</sup> content of the unsaponifiable lipide extract of the liver precipitable with digitonin could be recovered in the microsomal fractions. The experiments *in vivo* thus gave further support to the view derived from the experiments *in vitro* that microsomes are of prime importance in the synthesis of sterols. The fact that KCN and 2:4-dinitrophenol were without effect on cholesterol synthesis from acetate further suggested that mitochondria or mitochondrial functions inhibited by these agents are not involved in the process.

The coenzyme requirements of the Bucher preparations for cholesterol synthesis were shown to be Mg<sup>++</sup>, DPN and ATP [Frantz & Bucher (80); Bucher & McGarrah (82)]. The first step of cholesterol synthesis from acetate is, presumably, an activation reaction with CoA and ATP which is known to take place in the particle-free soluble protein fraction of liver homogenates.

It is relevant to examine here the evidence on the components of the enzyme system described by Rabinowitz & Gurin (83, 84) as being free of particles. These authors prepared a "mitochondrial" fraction from homogenates by centrifugation at 33,000 g for 40 min. and a "particle-free" supernatant obtained after centrifugation at 37,000 r.p.m. (approximately 100,000 g) in a Spinco centrifuge for 30 min. The "mitochondrial" fraction was then lysed with 1 volume of cold water for 1 hr. and the suspension of particles in water was then combined with 4 to 5 volumes of the "particle-free" supernatant and the mixture centrifuged again at 37,000 r.p.m. for 30 min. It is fairly certain that at 33,000 g not only mitochondria but an appreciable fraction of the microsomes are also sedimented and therefore in view of the detailed studies of Bucher & McGarrah (82) it is highly probable that the active principles in the preparations of Rabinowitz & Gurin (83, 84) are derived not from mitochondria but from microsomes. Attempts in the writer's laboratory to solubilize the microsomal enzymes concerned

in cholesterol synthesis, by lysis with water as described by Rabinowitz & Gurin (83) failed, the only difference being in our procedure that we centrifuged the preparations after lysis at a mean force of 105,000 g (40,000 r.p.m. Spinco rotor A40) for 60 min. Tchen & Bloch (85) in their experiments on the cyclization of squalene to sterol in rat liver homogenates, prepared according to Bucher, found that both the soluble protein and the particulate fractions were needed; although for the main purposes of their experiments the mitochondria and microsomes were not separated, it was ascertained by separate experiments that the microsomes rather than the mitochondria contained the enzymes of cyclization. The products of the cyclization were mainly cholesterol and a little lanosterol. Attempts to bring into solution the enzymes from the particulate fraction by freezing and thawing, by treatment with deoxycholate or ribonuclease, or acetone were not successful. However, exposure of the particles, suspended in the soluble supernatant of the homogenates, to ultrasonic oscillations (9 kc./sec.) for 15 sec. followed by centrifugation at 144,000 g for 40 min. gave a particle-free extract which, when combined with the soluble supernatant, promoted the cyclization of squalene to lanosterol; only minute amounts of cholesterol were formed by these particle-free preparations and the yield of sterol was only 2 to 5 per cent of that obtained with intact particles. If the homogenates of rat liver are made in a Waring blender, the ability of the preparations to convert acetate into sterol is lost, but the power to cyclize squalene to sterol is retained. Moreover, by varying the conditions under which the homogenizing is done, the preparations can be controlled to form either lanosterol or cholesterol, or both from squalene: (a) if nicotinamide and DPN were added during homogenizing the enzyme preparations converted squalene to cholesterol without accumulation of lanosterol; (b) if only nicotinamide was added during homogenizing, the preparations converted squalene into a mixture of lanosterol and cholesterol; (c) if DPN and nicotinamide were omitted during homogenizing and nicotinamide was added to the preparations immediately before the incubations, the principal product of the cyclization was lanosterol. Homogenates of pig liver made in the Waring blender are also active in forming sterol from squalene, but the process is not carried beyond the lanosterol stage. When thoroughly washed particles and dialysed soluble enzymes were used squalene was not converted into sterols unless reduced pyridine nucleotides were added, TPNH being more effective than DPNH. Tchen & Bloch (85) established also that the transformation of squalene to lanosterol is an aerobic process and that the oxygen in the sterol is derived from molecular oxygen and not from water; they called the liver enzyme catalysing the process "squalene-oxidocyclase I". It has been shown that molecular oxygen is the oxydant in a number of sterol oxygenation reactions and that TPNH is needed as a cofactor in the process [cf. review by Dorfman (3)].

Olson, Lindberg & Bloch (28) found that both the soluble supernatant fraction of rat liver homogenates and the microsomes were needed for the aerobic conversion of lanosterol into cholesterol. The activity of the soluble

fraction was destroyed by prolonged dialysis but could be partially restored by the addition of TPN and glucose-6-phosphate. Microsomes—in the absence of the supernatant fraction—supplemented with TPN, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were able to convert lanosterol to cholesterol at a rate about one-half of that seen with whole homogenates. On the other hand DPN, ethanol and alcohol dehydrogenase, ascorbate,  $\text{NaBH}_4$  or catechol were unable to activate the microsomes. Olson *et al.* (28) suggest that the participation of reduced TPN in oxidative reactions might be explained—in analogy with the behavior of phenolase [Mason, Fowls & Peterson (86)]—that the enzyme concerned is a metallo-protein and is active only in its reduced state.

Popják and his colleagues [Gould & Popják (62); Popják *et al.* (87, 88)] have studied the components of liver homogenates and the coenzymes required for cholesterol synthesis from mevalonic acid. These were generally found to be the same as those established [Bucher & McGarrahan (82)] for sterol synthesis from acetate. The mitochondria were definitely excluded from the process since supernatant liquids from homogenates obtained after centrifuging at a mean force of 20,000 g and 30,000 g for 60 min. were generally the most active preparations. Sterol synthesis from mevalonic acid could not be observed in the absence of microsomes, i.e., with supernatant liquids from homogenates centrifuged at a mean force of 105,000 g for 60 min. Nevertheless, such soluble preparations continued to synthesize squalene, although less efficiently than the complete system containing microsomes as well as soluble enzymes (62, 87, 88). As prepared freshly, the soluble protein fraction plus microsomes contained all the necessary factors for the synthesis of either squalene or cholesterol from mevalonic acid. Under anaerobic conditions squalene is formed exclusively, while in the presence of oxygen 75 to 90 per cent of the mevalonic acid utilized is found in sterol and the rest in squalene. The coenzyme requirements of the liver enzyme system were also determined by the use of dialysed soluble enzymes (redissolved after precipitation with ammonium sulphate) and washed microsomes. It was found that ATP, DPNH and TPN were needed in the presence of  $\text{Mg}^{++}$  for synthesis of squalene anaerobically. Neither of the pyridine nucleotides was specific, but for maximum enzyme activity both DPNH and TPN had to be added. It was shown further that an SH-compound, present mainly in the microsomes, is involved in the reactions leading to the formation of squalene from mevalonic acid. In anaerobic incubations this SH-compound is maintained in an active form, but in the presence of oxygen reducing agents (cysteine,  $\beta$ -mercaptoethylamine, glutathione or ascorbic acid) have to be added to the preparations. Synthesis from mevalonic acid of either squalene or of sterol did not occur in these preparations unless a reducing agent was introduced in addition to ATP, DPNH and TPN. The microsomal SH-compound is not involved in the cyclization of squalene to sterol as this could be achieved in these preparations in the presence of oxygen, DPNH and TPN. No definite role could be assigned to CoA in sterol or

squalene synthesis from mevalonic acid, although the addition of small amounts of CoA to the enzyme system consisting of dialysed soluble enzymes + washed microsomes usually produced 15 to 25 per cent stimulation of the synthetic activity. The dialysed preparations still contain some CoA as evidenced by activation of acetate after the addition of ATP alone. The optimum pH for sterol synthesis from mevalonic acid in the liver preparations lies between 7.3 and 7.5 which is higher than that found by Frantz & Bucher (80) for cholesterol synthesis from acetate (pH 7.1) in a similar enzyme system.

So far detailed reports by Tavormina and his colleagues on their experimental procedures and enzyme systems used have not appeared in spite of the fact that the announcement of their discovery was made at the end of the summer of 1956 at a Gordon Conference and published in two short notes in the autumn of that year.

The coenzyme requirements of the liver enzymes for squalene and sterol synthesis from mevalonic acid are very similar to those of the yeast enzyme system extracted with dilute phosphate buffer from baker's yeast by Amdur, Rilling & Bloch (41), which synthesizes squalene from mevalonic acid-2-C<sup>14</sup> both aerobically and anaerobically. Nevertheless there are distinct differences between the liver and yeast enzyme systems. The squalene synthesizing system could be precipitated from the original yeast extracts by 30 per cent saturation with ammonium sulphate. This is in contrast with the findings in the writer's laboratory (Popják, Gore & Gosselin, unpublished) with the liver enzymes in that the enzymic activity of the soluble liver fractions is confined entirely to the protein fraction precipitable between 30 and 60 per cent saturation with ammonium sulphate. Amdur *et al.* (41) found that either DPNH or TPNH, ATP, and Mn<sup>++</sup>, in preference to Mg<sup>++</sup>, were required by the yeast enzymes for squalene synthesis from mevalonic acid. The lack of specificity for the two pyridine nucleotides is similar to the observations made on the liver enzymes, although in the latter maximum activity, as measured by synthesis of either squalene or of sterol, was observed only when both were present. The yeast enzymes differ from those of the liver in one very significant point, namely, that the addition of a reducing agent was apparently not required even in the presence of oxygen. Since it has not yet been determined with certainty whether the SH-compound in the liver preparations is an enzyme or a coenzyme, no definite conclusion can be drawn as to whether the reactions in the two systems are identical or not. No requirement for CoA could be shown in the yeast enzymes, even after treatment with Norite-A. The distinct need for Mn<sup>++</sup> by the yeast enzymes could not be shown with freshly prepared liver preparations; however, microsomes, which after several weeks' (7 to 11 weeks) storage at -15° lose much of their activity (50 per cent loss in 4 weeks and 80 to 90 per cent inactivation after 8 weeks) may be almost fully reactivated by the addition of Mn<sup>++</sup> (Gosselin, Gore & Popják, unpublished).

The use of mevalonic acid as substrate for squalene and sterol synthesis



has undoubtedly greatly facilitated the study of the enzyme reactions of squalene and sterol synthesis; nevertheless it will require much effort to disentangle the multiplicity of reactions involved in the entire process. Far more is known at present about the squalene→sterol transformation than about the reactions leading to the formation of squalene. Up to now, no evidence has been presented as to the synthesis of mevalonic acid in enzyme systems, although presumably it is a by-product of alcoholic fermentation. The main difficulty in demonstrating the accumulation of mevalonic acid in an enzyme system involved in squalene or sterol synthesis is no doubt due to the great efficiency with which mevalonic acid is utilized. For example, each millilitre of the crudest liver enzyme preparations (supernatants of homogenates after centrifugation at 10,000 g for 25 min.) used in the writer's laboratory will convert under optimal conditions of coenzyme concentrations 0.5 to 1.0  $\mu$ mole of mevalonic acid into squalene plus cholesterol in 3 hr. In comparison only a few millimicromoles of acetate are utilized by these preparations [cf. also Frantz & Bucher (80); Bucher & McGarrahan (82)]. If it is assumed that the synthesis of sterol from acetate occurs through mevalonic acid as an obligatory intermediate, it is apparent that the formation of mevalonic acid from acetate must be a severely rate-limiting step in squalene or sterol biosynthesis. The observation of Wright *et al.* (65) that pretreatment of liver homogenates with ribonuclease blocks the utilization of mevalonic acid for cholesterol synthesis will no doubt be of great assistance in studying the biosynthesis of mevalonic acid. The conversion of mevaldic to mevalonic acid was shown in such a blocked system (65).



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## AMINO ACID METABOLISM<sup>1,2</sup>

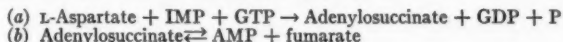
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Since certain aspects of protein metabolism are discussed elsewhere in this volume, this review is confined to the metabolism of the individual amino acids. Emphasis has been placed on reactions which are well defined from enzymatic studies and on the role of specific amino acids in the synthesis of more complex nonprotein cell constituents.

### ASPARTIC ACID

A particularly interesting facet of aspartate metabolism is the role of this amino acid in the synthesis of purines and their derivatives. Subsequent to the finding that the conversion of IMP to AMP requires L-aspartic acid and a high energy phosphate source (1), Lieberman (2, 3) has established adenylosuccinate as an intermediate, as follows:



Reaction *a*, catalyzed by "adenylosuccinate synthase" from *Escherichia coli* B, requires GTP. On the basis of studies with IMP-6-O<sup>18</sup>, Lieberman has suggested that 6-phosphoryl-IMP may first be formed and that displacement of the phosphate group by aspartate may yield adenylosuccinate. This product is then cleaved by "adenylosuccinase" in an aspartase-type cleavage according to Reaction *b* (4, 5). Carter (6) has recently provided proof for the structure of adenylosuccinate by comparing its properties with those of the aglycone (adeninosuccinic acid), and Hampton (7) has synthesized the corresponding nucleoside by an unambiguous route and shown its identity with the compound obtained upon treating Carter's adenylosuccinate with phos-

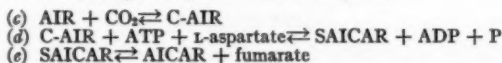
<sup>1</sup> The survey of the literature pertaining to this review was completed in November, 1957.

<sup>2</sup> The following abbreviations are used in this chapter: ATP, ADP, and AMP for adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate, respectively; CoA for coenzyme A; acyl CoA for thiol ester of coenzyme A; DPN (or DPN<sup>+</sup>) and DPNH for oxidized and reduced diphosphopyridine dinucleotide, respectively; FAD for flavin adenine nucleotide; FMN for flavin mononucleotide; GTP and GDP for guanosinetriphosphate and guanosinediphosphate, respectively; HIV for  $\beta$ -hydroxy-isovaleric acid; HMG for  $\beta$ -hydroxy- $\beta$ -methylglutaric acid; IMP and ITP for inosinic acid and inosine triphosphate, respectively; P for orthophosphate; PP for pyrophosphate; R-5-P for ribose-5-phosphate; and TPN (or TPN<sup>+</sup>) and TPNH for oxidized and reduced triphosphopyridine nucleotide, respectively. The letter U, as in proline-U-C<sup>14</sup>, is used to designate a uniformly labeled compound.

<sup>3</sup> The authors are indebted to Dr. Halvor N. Christensen and Dr. G. Robert Greenberg for helpful discussions.

phataases. Adenylosuccinate has recently been isolated from mammalian liver (8) and *Penicillium chrysogenum* (9), and adeninosuccinate [6-(succino-amino)-purine] (10, 11) and its riboside (11) have been obtained from *Neurospora* mutants. Tsuyuki & Idler (12) have identified an additional adenylosuccinate derivative which contains sulfate (as the ribose-5'-phosphosulfate) and a peptide residue containing glutamic acid, serine, and perhaps other amino acids. The structure of the peptide and the manner in which it is linked to the nucleotide are still under investigation.

Reactions analogous in some respects to those described above have been established by Lukens & Buchanan (13) in accounting for the origin of N-1 of the purine nucleus from the amino group of aspartic acid. The following reaction sequence is catalyzed by avian liver enzymes:



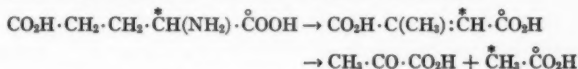
Reaction *c* represents the carboxylation of 5-aminoimidazole ribotide (AIR) in the presence of carbon dioxide to furnish 5-amino-4-imidazolecarboxylic acid ribotide (C-AIR). This product then undergoes an ATP-catalyzed condensation with aspartate to yield 5-amino-4-imidazole-N-succinocarboxamide ribotide (SAICAR) according to Reaction *d*. The similarity in the cleavage of the succinocarboxamide ribotide to fumarate and 5-amino-4-imidazolecarboxamide ribotide (AICAR) and in the cleavage of adenylosuccinate is striking, and Buchanan and his associates (14) have indeed shown that both are catalyzed by adenylosuccinase. Studies with adenine-requiring *Neurospora* mutants have supported the interesting conclusion that the two distinct aspartate-requiring steps in AMP synthesis are catalyzed by a single enzyme and are, therefore, apparently under the control of a common genetic unit. Gots & Gollub (15) have reached a similar conclusion upon finding that certain mutants of *E. coli* and *Salmonella typhimurium* are lacking this bifunctional cleavage enzyme.

Other papers have provided information on several different aspects of aspartate metabolism. Monder & Meister (16) have stated in a preliminary report that cell-free *Neurospora* extracts catalyze the reaction of  $\alpha$ -ketosuccinamic acid with glutamine to furnish asparagine. This observation recalls earlier studies of others which suggest that asparagine synthesis in mammalian cells involves the utilization of glutamine amide nitrogen. Tallan (17), in an extension of earlier work which demonstrated the occurrence of N-acetyl-L-aspartic acid in brain (18), has reported that this compound occurs at a concentration of 80 to 110 mg. per cent in mammalian and avian brain tissues, but is not detectable in the brains of certain lower animals. The metabolic importance of this interesting compound, which occurs throughout the central nervous system in mammals, is apparently completely unknown. Recent reports on aspartase have been concerned with the presence of this enzyme in *Bacterium cadaveris* (19) and higher plants (20) and with its purification from *Propionibacterium peterssonii* and *Pseudomonas fluorescens* (21).

## GLUTAMIC ACID AND GLUTAMINE

*Glutamic acid metabolism.*—Barker *et al.* (22), in continuation of earlier work, have described a new pathway of D- or L-glutamate decomposition in extracts of *Clostridium tetanomorphum* leading to the formation of ammonia and mesaconic acid. This transformation apparently involves a cleavage between C-2 and C-3 of glutamate with formation of a new bond between C-2 and C-4. In the presence of cysteine and ferrous ions mesaconate is converted to *d*-citramalate, which in turn is cleaved with the formation of pyruvate and acetate. Since these reactions are reversible, acetate, pyruvate, and ammonia can lead to the formation of D- or L-glutamate in this microorganism. Adler (23) has recently carried out studies on the metabolism of branched 5-carbon dicarboxylic acids which may possibly be related to the findings just described. In a preliminary report he has described the following reaction sequence in liver mitochondrial extracts: itaconate  $\xrightarrow{\text{CO}_2}$  itaconyl CoA (tricarboxylic acid)  $\rightarrow$   $\alpha$ -ketoglutarate  $\rightarrow$  glutamate. Similar extracts from biotin-deficient rats are less effective in catalyzing the carboxylation step. Some of these reactions are analogous to those already known for the conversion of senecioate to acetoacetate and acetyl CoA in animal tissues (24). It remains to be established whether the enzymes of leucine metabolism will act on these interesting branched-chain compounds related to glutamate metabolism.

It appears that two isotopic studies which have yielded unexpected findings may be explained by the Barker scheme, as follows:



First, Fry (25) has found that washed cell suspensions of *C. tetanomorphum* effect the conversion of glutamate-1-C<sup>14</sup> to acetate-1-C<sup>14</sup> and butyrate-1,3-C<sup>14</sup> and unlabeled carbon dioxide. Second, Koeppe and his associates (26) have shown that the distribution of C<sup>14</sup> in aspartic acid, alanine, and serine, after the administration of glutamate-2-C<sup>14</sup> to rats, cannot be explained by the known route leading directly to  $\alpha$ -ketoglutarate and the citric acid cycle. On the other hand, these amino acids appear to have been derived from methyl-labeled acetate. Another pathway of glutamate metabolism, possibly via the formation of  $\gamma$ -aminobutyrate, leads to the formation of pyocyanine in *Pseudomonas aeruginosa* (27). This blue pigment has antibiotic activity toward Gram-positive bacteria, probably by inhibiting the oxidation of certain keto acids (28).

*Glutamine metabolism.*—Readers are referred to a comprehensive review of glutamine metabolism by Meister (29). A recent contribution to our understanding of the glutamine synthetase reaction has been provided by Boyer & Fromm (30), who propose that ammonia is added to enzyme-bound glutamate. ATP is then pictured as effecting the synthesis of a phosphorylated intermediate which decomposes to glutamine and phosphate. Sachs & Waelsch (31) have carried out chemical and enzymatic studies which show

that S- $\alpha$ - and S- $\gamma$ -glutamylglutathione are highly reactive compounds, but there is as yet no proof for thiol esters of glutamic acid as biological intermediates in such reactions as glutamine synthesis.

Glutamine plays a role in many important metabolic pathways. Several mammalian cells are known to require this compound for survival and growth in tissue culture (32), and Levintow and his associates (33, 34, 35) have now shown that glutamine and glutamic acid act independently in protein synthesis in HeLa cells. Similarly, Rabinovitz *et al.* (36) have provided evidence that glutamine acts as a limiting component in protein synthesis in Ehrlich ascites carcinoma cells, and Eagle *et al.* (37) have shown that five lines of human tissue culture cells require 12 amino acids, including glutamine.

The manner in which the amide nitrogen of glutamine gives rise to N-3 and N-9 of the purine nucleus has been the subject of earlier reports (38 to 43). N-9 is furnished by the following over-all reaction: Glycine + glutamine + 5-phosphoribosylpyrophosphate  $\rightarrow$  glycinamide ribotide + glutamate. Since synthetically prepared 5-phosphoribosylamine can replace glutamine and 5-phosphoribosylpyrophosphate, the over-all reaction is believed by Goldthwait (44) to occur by these steps:

- (a) Glutamine + 5-phosphoribosylpyrophosphate  
 $\rightarrow$  phosphoribosylamine + glutamate + PP
- (b) Phosphoribosylamine + glycine + ATP  $\rightleftharpoons$  glycinamide ribotide + ADP + P

Since PP<sub>2</sub> was observed not to exchange with the phosphoribosylpyrophosphate, the mechanism by which phosphoribosylamine is formed in Reaction *a* is not well understood (44). Reaction *b* appears to be reversible, and may involve the formation of an activated form of glycine such as glycyI phosphate (45). In a study of the mechanism of origin of N-3 of the purine ring, Buchanan and his associates (46, 47) have established the following reactions:

- (c)  $\alpha$ -N-formylglycinamide ribotide + glutamine + ATP  
 $\rightarrow$   $\alpha$ -N-formylglycinamide ribotide + glutamate + ADP + P
- (d)  $\alpha$ -N-formylglycinamide ribotide  $\xrightarrow{\text{ATP}}$  5-aminoimidazole ribotide

The enzymes catalyzing these individual reactions have been separated, and azaguanine and 6-diazo-5-oxo-L-norleucine have been shown to behave as competitive inhibitors for glutamine in blocking Reaction *c* (48). It should be noted that the other glutamine-requiring step in purine biosynthesis (Reaction *a*) is inhibited by the glutamine antagonist, azaserine (44). Skipper and his associates (49, 50) have studied the inhibitory effect of azaserine on purine synthesis in *E. coli* and animal tissues and have reported that this compound blocks the conversion of  $\alpha$ -N-formylglycinamide ribotide to 5-amino-4-imidazolecarboxamide ribotide. This finding is in general agreement with the conclusions reached in the enzymatic studies just described.

Other metabolic roles of glutamine include its reaction with phenylacetyl CoA in human tissues to yield phenylacetyl glutamine, as shown by Moldave & Meister (51, 52, 53), and its participation in the conversion of fructose-6-



phosphate to glucosamine-6-phosphate (54 to 58). Although earlier studies (59, 60) have demonstrated that the addition of glutamine to the diet of laboratory rats reduces their voluntary alcohol intake, the metabolic significance of this interesting observation is still unknown (61).

Goldstein, Richterich-van Baerle & Dearborn (62) have reported that in acute acidosis the glutaminase I (phosphate-activated) activity of guinea pig kidney is increased, whereas in acute alkalosis both glutaminase I and glutaminase II (pyruvate-activated) activity are significantly increased. The authors state that their findings are suggestive of enzyme induction, but no information is yet available on the quantity of enzyme present under these circumstances. This group (63, 64) has also studied the properties of these two enzymes in guinea pig kidney preparations. Klingman & Handler (65, 66) have partially purified the phosphate-activated glutaminase of dog kidney and obtained it relatively free of the glutamine-dependent glutathionase activity of cruder preparations. Phosphate and borate were found to "activate" the enzyme by protecting against the rapid inactivation which occurs in the absence of anions. Ammonia, unlike glutamate, was found to be reincorporated into glutamine when incubated with glutamine, phosphate, and the enzyme. Azaserine was found to inhibit the enzymatic hydrolysis of glutamine.

Glutaminases have now been demonstrated in diverse sources, such as *Endamoeba histolytica* (67), pleuropneumonia-like organisms (68), and mitochondria of HeLa cells grown in tissue culture (69). Blumson (70) has studied glutamine hydrolysis by rat brain particles and concludes that the claim that either IMP or a mixture of ITP and creatine phosphate stimulates the reaction (71) may be accounted for by the presence in these compounds of low concentrations of phosphate and sulfate. Benzinger & Hems (72) have made calorimetric measurements of glutamine hydrolysis and have calculated that the free energy change ( $\Delta F^\circ_{310}$ ) is  $-3430$  cal. per mole. This figure is in accord with the general conclusion that the glutaminase reaction is practically irreversible.

#### PROLINE AND HYDROXYPROLINE

Further information on the following pathway for the interconversion of glutamic acid and proline in animal tissues and microorganisms (73, 74, 75) has been obtained during the past year: Glutamic acid  $\rightleftharpoons$  glutamic- $\gamma$ -semialdehyde  $\xrightleftharpoons{\text{(nonenzymatic)}}$   $\Delta^1$ -pyrroline-5-carboxylic acid  $\rightleftharpoons$  proline. Strecker (76), in an attempt to obtain information on the mechanism of the reduction of the  $\gamma$ -carboxyl group of glutamate, has observed that the addition of various simple substrates, such as lactate, increases  $\Delta^1$ -pyrroline-5-carboxylic acid formation from L-glutamate or L-glutamine by washed cells of an *E. coli* mutant. The addition of AMP or ADP provided further stimulation, whereas ATP was inhibitory. The reason for these effects is not yet known. A lag in the reduction of glutamate, until endogenous proline was metabolized, was regarded as an indication of control by a feedback mech-

anism (76). Smith (77) has observed that this same pathway occurs in pleuropneumonia-like organisms and that the conversion of glutamate to  $\Delta^1$ -pyrroline carboxylic acid requires magnesium ions and ADP or ATP. He has also found that TPNH is required for the reduction of the cyclic intermediate to proline. Smith & Greenberg (78, 79) have partially purified from rat liver the DPN-dependent "glutamic semialdehyde reductase," which presumably reduces pyrroline carboxylic acid to proline; a similar enzyme has been described in *Neurospora* by Yura & Vogel (80).

Kanazir (81) has described the above pathway as well as the conversion of ornithine to proline in *S. typhimurium* mutants, and Maruyama & Nomurra (82) have provided the suggestion that a soil *Pseudomonas*, cultivated on a medium containing pyrrolidone carboxylic acid, may convert this compound to pyroglutamic acid ( $\Delta^1$ -pyrroline-5-one-2-carboxylic acid). In extension of an earlier report on proline reduction in *Clostridium sticklandii* (83), Stadtman & Elliott (84) have described reactions leading to the formation of  $\delta$ -aminovaleric acid. Two distinct protein fractions are necessary for the complete reduction of DL-proline. One of these fractions is a racemase which rapidly converts either optical isomer of proline to the racemic mixture. The other fraction catalyzes the reduction of D-proline to  $\delta$ -aminovaleric acid and has no activity on L-proline.

The conversion of hydroxy-D- and allohydroxy-D-proline to pyrrole-2-carboxylic acid in mammalian tissues has been demonstrated by Letellier & Bouthillier (85) and by Radhakrishnan & Meister (86). The latter investigators have found that D-amino acid oxidase converts these hydroxy-D-amino acids to  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylic acid, which then undergoes spontaneous dehydration to form pyrrole carboxylic acid. This same product was also obtained by the transamination of  $\gamma$ -hydroxyornithine with glyoxalate, pyruvate, or  $\alpha$ -ketoglutarate (86), presumably with the intermediate formation of  $\alpha$ -keto- $\gamma$ -hydroxy- $\delta$ -amino valeric acid and  $\Delta^1$ -pyrroline-4-hydroxy-2-carboxylic acid. However, since the D amino acids which undergo these reactions are not known in mammalian tissues, the significance of the reactions remains to be determined.

Adams (87) has demonstrated that soluble extracts of a soil bacterium grown on hydroxy-L-proline generate L-glutamate from this substrate. Allohydroxy-D-proline also yields glutamate under certain conditions, owing to the presence of a highly active epimerase in the crude extracts, but a preliminary search revealed no epimerase in animal tissue extracts. Wolf *et al.* (88) have found that subsequent to the injection of hydroxy-DL-proline into rats the alanine of the tissue proteins is highly radioactive and glutamic and aspartic acids somewhat less so. This may provide evidence for glutamate formation from hydroxyproline in animals, as in bacteria (87), but it is not clear, as yet, whether still another new pathway to alanine is thus indicated.

The origin of the hydroxyproline and hydroxylysine residues in proteins continues to be an absorbing problem. Mitoma & Smith (89) have noted that fibroblasts of subcutaneously implanted polyvinyl sponges catalyze the con-

version *in vitro* of  $C^{14}$ -labeled L-proline to collagen containing labeled hydroxyproline. This is, of course, in general accord with the conclusion of Stetten (90) that hydroxyproline is not used directly for protein synthesis but is generated from proline in a bound or combined form.

There have been two further reports on the possible role of ascorbic acid in collagen synthesis. In a study of skin regeneration in normal and scorbutic guinea pigs, Gould & Woessner (91) have concluded that impaired hydroxyproline formation may be one of the earliest manifestations of ascorbic acid withdrawal. The rapid production of hydroxyproline in scorbutic animals after administration of this vitamin may be due to the conversion of an accumulated pool of protein rich in proline and glycine to a more immediate collagen precursor rich in hydroxyproline. As evidence for this conclusion, the appearance of hydroxyproline was accompanied by a decrease in bound, noncollagen proline of the granulation tissue. Such a role for ascorbic acid may also account for the observations of Robertson *et al.* (92), who injected proline- $U-C^{14}$  into guinea pigs and found the specific activity of hydroxyproline from the granuloma collagen of scorbutic animals to be reduced to 25 per cent of that in the normal animals.<sup>3</sup> Sinex & Van Slyke (93) have studied the role of dietary lysine-6- $C^{14}$  as a precursor of the hydroxylysine residues of skin collagen, and have found a similarity in the mechanism of biosynthesis of hydroxyproline and hydroxylysine.

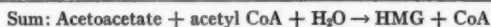
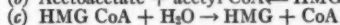
A third hydroxyamino acid, 5-hydroxypipicollic acid, has been identified in dates (94), and the configuration has been established by Witkop & Foltz (95). The biochemical role of this interesting compound is as yet unknown.

#### LEUCINE METABOLISM AND ADENYLIC ANHYDRIDES

*Leucine metabolism.*—Evidence for common intermediates in leucine catabolism and in the biosynthesis of isoprenoid compounds from acetate has accumulated in the past few years (96 to 101). Indeed, leucine may function in the intact organism as a precursor of both steroids and proteins. Recent studies on the mechanism of the branching reaction represented by the condensation of "acetate" and "acetoacetate" indicate that two variations may be possible. The first of these reactions (Reaction *a*), catalyzed by  $\beta$ -hydroxy- $\beta$ -methylglutaric acid (HMG) condensing enzyme, has been clearly shown by Rudney & Ferguson (102, 103, 104) to occur in rat liver, beef liver and yeast.



The free CoA liberated in this reaction is derived from acetyl CoA. Alternatively, it has been suggested that the reversal of the HMG CoA cleavage reaction (Reaction *b*) may provide an alternate branching mechanism (105).



Although the equilibrium of Reaction *b* is to the left, extracts of liver, kidney, and brain contain a deacylase for  $\beta$ -hydroxy- $\beta$ -methyl glutaryl CoA (105) catalyzing Reaction *c*, which would be expected to couple with Reaction *b*, as shown. The free energy change in Reaction *a* would probably be about the same as in the sum of Reactions *b* and *c*.

A striking development in steroid biogenesis has originated with the identification of mevalonic acid ( $\beta$ ,  $\delta$ -dihydroxy- $\beta$ -methylvaleric acid) as a new acetate-replacing factor for *Lactobacilli* (106, 107). Tavormina & Gibbs (108) have demonstrated that mevalonic acid is a remarkably efficient chole-

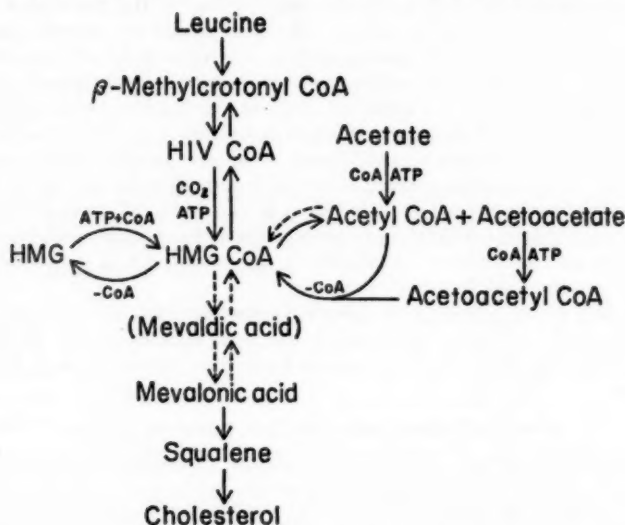


FIG. 1. Isoprenoid synthesis from acetate and leucine.

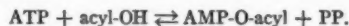
sterol precursor and that the carboxyl carbon is lost during this conversion. The formation of squalene from *dl*-mevalonic acid-2- $\text{C}^{14}$  has been demonstrated in rat liver homogenates by Ditur, Gurin & Rabinowitz (109), and in particle-free yeast extracts by Amdur, Rilling & Bloch (110). The latter investigators found that this conversion requires the presence of manganous ions, ATP, and a pyridine nucleotide; they also performed an experiment with tritium-labeled mevalonic acid which appears to rule out mevaldic acid ( $\beta$ -hydroxy- $\beta$ -methylglutaraldehydic acid) as an obligatory intermediate in the conversion of mevalonic acid to squalene. Shunk *et al.* (111) have prepared *dl*-mevaldic acid and have found that it is a cholesterol precursor, as judged by its ability to suppress the incorporation of acetate- $\text{C}^{14}$  into cholesterol, but that it is only 1/200 as active as *dl*-mevalonic acid as a growth factor for *Lactobacillus acidophilus*. Mevalonic acid lactone-2- $\text{C}^{14}$  has been

shown by Gould & Popják (112) to yield labeled cholesterol in the intact mouse as well as in liver homogenates; presumably the lactone first undergoes hydrolysis to the free acid. Our present picture of these conversions is shown in Figure 1.

Although this scheme appears to be applicable to the synthesis of other isoprenoid substances such as the triterpene eburicoic acid (113, 114) and carotene (115, 116), Zabin (117) has reported that labeled acetate is incorporated into lycopene by a somewhat different route.

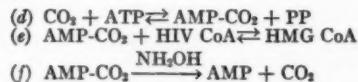
An indication that a second amino acid plays a role in steroid biogenesis is provided by the report that  $C_{28}$  of ergosterol is derived from the methyl group of methionine (118) and  $C_{28}$  of eburicoic acid from formate (119).

*Adenylic anhydrides in amino acid metabolism.*—Evidence has recently emerged that various molecules with acidic groups are activated by ATP with the formation of a mixed anhydride with the phosphate group of adenylic acid as follows:



This type of activation has been discussed by Kornberg (120) in a review on pyrophosphorylases and phosphorylases in biosynthetic reactions, to which readers are referred. In this type of activation reaction the individual enzymes appear to be remarkably specific, and the acyl-AMP compound generally does not accumulate readily. Evidence for these reactions is gained from exchange studies or by degradation of the reactive intermediate in the presence of hydroxylamine.

The carbon dioxide-fixing step in leucine metabolism may be formulated as follows (121, 122):

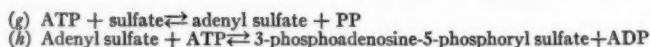


The bicarbonate-dependent cleavage of ATP (Reaction *d*) is catalyzed by extracts of bacteria, yeast, and animal tissues, and can be measured when coupled with Reaction *e* (catalyzed by  $\beta$ -hydroxyisovaleryl CoA carboxylase) or with Reaction *f*, which represents the non-enzymatic decomposition of adenylic carbonate ( $\text{AMP-CO}_2$ ) in the presence of hydroxylamine. Flavin *et al.* (123, 124) have recently described a different enzyme, fluorokinase, which catalyzes the fluoride- and bicarbonate-dependent cleavage of ATP to ADP and fluorophosphate, and have suggested that fluorokinase may generate a compound such as carbonyl phosphate as a carboxylating agent in the conversion of propionyl CoA to methylmalonyl CoA. The carbon dioxide-activating enzyme which catalyzes Reaction *d* has been isolated in crystalline form from pig heart and found to require zinc ions for maximal activity. This enzyme is present in biotin deficiency, whereas the carboxylase is completely lacking (121). The observation of Jacobsohn & Corley (125) that the formation of cholesterol from  $\beta$ -methylcrotonic acid is diminished in liver slices from biotin-deficient rats is readily accounted for by these results.

The report that the incorporation of  $C^{14}$ -labeled carbon dioxide into glutamate is stimulated by the administration of leucine (126) can probably be satisfactorily explained by the intermediate formation of carboxyl-labeled acetoacetate from leucine (127, 128). Hendler (129) has found that  $C^{14}O_2$  fixation in hen oviduct tissue leads to a steam-volatile compound not identical with the known leucine metabolites.

Although extensive evidence supports the metabolic role of adenylic anhydrides of fatty acids (130 to 134), pantoic acid (135, 136), luciferin (137), and phenylacetic acid (138), these compounds, like adenylic carbonate, apparently cannot be accumulated directly by the action of the activating enzymes. The most likely explanation appears to be that the equilibrium lies far over toward ATP, thereby suggesting that the acyl-adenylate bond is of considerably "higher energy" than is the terminal pyrophosphate bond of ATP.

In contrast to the activating reactions so far discussed, sulfate activation, as shown by Robbins & Lipmann (139) and by Bandurski *et al.* (140), requires the coupled action of two enzymes. Adenylic sulfate, the product of Reaction g, is converted to 3-phosphoadenosine-5-phosphoryl sulfate as follows:



According to a brief report, Segal (141) has also studied sulfate activation and arrived at similar conclusions.

Although amino acid activation is discussed elsewhere in this volume under the topic of protein synthesis, mention should also be made here of the enzymatic synthesis of adenylic amino acids. The formation of amino acid hydroxamates and the amino acid-dependent exchange between pyrophosphate and ATP in liver preparations have been described by Hoagland *et al.* (142, 143), in bacterial extracts by De Moss & Novelli (144, 145), and in plant preparations by Davis *et al.* (146). The enzymes which activate amino acids, like the other activating enzymes already described, are highly specific. Berg (147, 148) has described a yeast enzyme which incorporates  $P^{32}$ -labeled pyrophosphate into ATP in the presence of methionine, and Davie *et al.* (149) have obtained a tryptophan-activating enzyme from beef pancreas. Furthermore, Holley (150) has demonstrated an alanine-dependent, ribonuclease-inhibited conversion of AMP to ATP; Cole, Coote & Work (151) have found a serine-threonine-activating enzyme in guinea pig pancreas; and Schweet (152) has isolated a tyrosine-activating enzyme from hog pancreatic extracts.

#### ISOLEUCINE AND VALINE

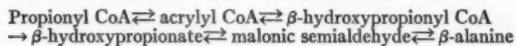
Methylethyl ketone has been identified by Tsao & Pfeiffer (153) as a new "ketone body" occurring in human urine. They propose that it is formed by decarboxylation of  $\alpha$ -methylacetoacetate, which is known to occur in the form of its CoA ester as one of the intermediates in isoleucine metabolism in mammalian enzyme systems (154). The detection of methylethyl ketone in



urine suggests that the  $\alpha$ -methyl analogues of acetoacetate and  $\beta$ -hydroxybutyrate may also prove to be excretion products. The role of  $\alpha$ -aminobutyric acid as an isoleucine precursor in *Neurospora* (155, 156) has been confirmed by Herrmann & Fairley (157) in a study of a pyrimidineless mutant.

Recent enzymatic studies (158) have established that isobutyryl CoA, an intermediate in valine metabolism, undergoes only two reactions which would be predicted by analogy to the metabolism of straight chain fatty acids: dehydrogenation to methacrylyl CoA and subsequent hydration (by enoyl hydratase) to form  $\beta$ -hydroxyisobutyryl CoA. Enzymatic hydrolysis of the latter compound by a specific deacylase (159) and attack of the resulting free  $\beta$ -hydroxyisobutyrate by a highly specific DPN-dependent dehydrogenase (160) furnish methylmalonic semialdehyde. The occurrence of these enzymes in a variety of microorganisms and animal tissues indicates that this is a general pathway for the metabolism of valine. The mechanism by which methylmalonic semialdehyde is further metabolized is not yet certain, but earlier isotopic studies have shown that propionate and carbon dioxide are the probable end products of valine metabolism. The known metabolic relationship of methylmalonate to propionate (123, 161) and to valine (162) has led to the suggestion that methylmalonic semialdehyde may be oxidized directly to methylmalonic acid or a closely related derivative (163).

The investigations on valine metabolism have led to the proposal of the following analogous  $\beta$ -oxidative pathway for propionate degradation, in which all of the steps except the dehydrogenation of propionyl CoA have been demonstrated in animal enzyme systems (159):



Giovanelli & Stumpf (164) have provided isotopic evidence for this new pathway in peanut mitochondria.  $\beta$ -Hydroxypropionate is not converted to  $\beta$ -alanine in their preparations, but is oxidized to Krebs cycle intermediates, thereby indicating the importance of these reactions in the complete oxidation of propionate.

In continuation of earlier work (165), Vagelos & Stadtman (166) have purified a *Clostridium propionicum* enzyme which catalyzes the addition of ammonia to acrylyl pantotheine to form  $\beta$ -alanyl pantotheine. This reaction could not be detected in several strains of *Pseudomonas*, but when grown on propionate this organism contains an enzyme which converts acrylyl pantotheine to a product tentatively identified as lactyl pantotheine. These reactions leading to  $\beta$ -alanine formation from propionate provide an alternate synthetic route to the pathway from pyrimidines (167 to 172).

Umbarger, Brown & Eyring (173) have reported that  $\alpha$ -acetolactate, proposed earlier as an intermediate in valine biosynthesis (174), is accumulated by a valineless *E. coli* mutant. They have further shown that  $\alpha$ -acetolactate is converted to  $\alpha,\beta$ -dihydroxy-isovaleric acid, a known valine precursor (175), and gives rise to valine in the bacterial protein. Recent studies

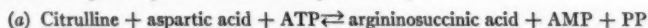


(176) have confirmed the role of valine in penicillin biogenesis (177,178) and have ruled out  $\beta$ -hydroxyvaline as a possible intermediate (179). The inhibitory effect of lysine on penicillin synthesis in *P. chrysogenum* (180) may possibly explain the observation that lysineless strains of *Penicillium notatum* show defective penicillin synthesis when grown in the presence of lysine (181).

The role of  $\alpha$ -ketoisovalerate as a precursor of pantoic acid has been clarified by the report of McIntosh *et al.* (182) of an *E. coli* enzyme which catalyzes the condensation of this keto acid with formaldehyde to furnish ketopantoate ( $\alpha$ -keto- $\beta$ , $\beta$ -dimethyl- $\gamma$ -hydroxybutyric acid). In agreement with earlier experiments, Womack, Snyder & Rose (183) have found that D-valine at 1 per cent of the diet, unlike L-valine, is incapable of supporting the growth of weanling rats. At twice this level D-valine supports slow growth, but this effect is suppressed when the diet contains DL-leucine in place of L-leucine. Whether this effect is due to a competitive inhibition of D-valine inversion, as proposed by Wretling (184), or is to be explained otherwise remains to be determined.

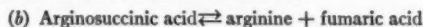
#### ARGININE METABOLISM AND UREA SYNTHESIS

*Urea synthesis.*—The important discovery by Ratner and her associates in 1953 that argininosuccinic acid is an intermediate in the conversion of citrulline to arginine has been extended in a recent study. Ratner & Petrack (185) have now shown the nature of the two enzymes that are involved in the generation of this intermediate from citrulline in mammalian liver. Reaction *a* is catalyzed by one of these enzymes



and the other has been identified as pyrophosphatase, which enhances argininosuccinate formation by removing pyrophosphate. These investigators have demonstrated the reversibility of Reaction *a* in experiments which employed kidney or liver enzymes relatively free of pyrophosphatase. The stoichiometry of the reaction corresponds to the equation given, and, as expected, the conversion of argininosuccinic acid to citrulline requires the addition of both AMP and PP.

The formation of arginine according to Reaction *b*, catalyzed by argininosuccinase, is well established in animal tissues:

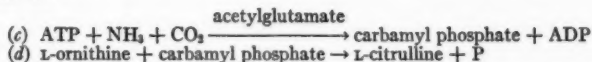


Fincham & Boylen (186) have described *Neurospora crassa* mutants which require arginine as a nutrient but which fail to respond to citrulline. Extracts of these mutants, in contrast to those of the wild type, lack argininosuccinase. In contrast, Suzuki *et al.* (187) have found an arginine-requiring *E. coli* mutant which does not respond to citrulline but is unable to form argininosuccinic acid. Possibly the enzyme catalyzing the citrulline-aspartic condensation is absent from this type of mutant.

A recent study has also added to our knowledge of arginase. Although this enzyme has generally been considered to be bound to nuclear and micro-

somal cell fractions, Rosenthal *et al.* (188) have shown, with the use of improved techniques, that this enzyme occurs in the soluble fraction of the cytoplasm. This observation emphasizes that the distribution of an enzyme among the cellular components may be determined by the nature of the medium employed for homogenization of the tissue.

Despite major contributions in recent years to our understanding of the ornithine-citrulline conversion, some very interesting problems remain under investigation. Hall, Metzenberg & Cohen (189) have isolated and characterized the naturally occurring stimulator of citrulline biosynthesis as N-acetylglutamate. This substance was isolated in crystalline form from beef liver and from yeast extract, where it occurs at concentrations of 3 p.p.m. and 200 p.p.m., respectively. Acetyl glutamate is known to be required for carbamyl phosphate synthesis (Reaction *c*) in mammalian liver extracts, but not in bacterial preparations. Metzenberg, Hall & Cohen (190) have studied the role of this cofactor in a liver enzyme system in which carbamyl phosphate synthesis was assayed by phosphate liberation in the presence of an excess of ornithine-carbamyl phosphate transcarbamylase, which catalyzes Reaction *d*:



At rate-limiting concentrations of acetylglutamate, two moles of ADP and two moles of P were found to be formed per mole of citrulline generated, but in the presence of an excess of acetylglutamate more than two moles of P were liberated per mole of citrulline. The authors indicate that such data may provide evidence for the existence of an intermediate prior to carbamyl phosphate. In studying the same problem, Grisolia & Towne (191) have proposed the formation of a phosphorylated intermediate by a reaction between ATP and acetylglutamate. In extension of earlier work (192) they observed that hydroxylamine increased P liberation in the mammalian citrulline-forming system, but not in the bacterial system, and that preincubation of their enzyme preparations with acetylglutamate and ATP increased citrulline synthesis.

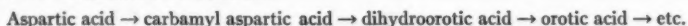
The role of biotin in citrulline synthesis has also come under recent investigation. Estes, Ravel & Shive (193) have noted that extracts prepared from biotin-deficient *Streptococcus lactis* cells have a diminished ability to convert carbamyl phosphate and ornithine to citrulline. This conclusion and the findings of others (194, 195) indicate that biotin may play a role in both the formation and transfer of the carbamyl group in citrulline synthesis. Another factor to be considered in the regulation of these reactions is the inhibition by arginine of ornithine transcarbamylase synthesis. Gorini & Maas (196, 197) have described this negative feedback effect and have reported that wild type *E. coli* cells have a potential for synthesizing this enzyme greatly in excess of that reached under normal conditions where inhibitory concentrations of arginine accumulate.

Reichard (198) has obtained highly purified preparations of ornithine-

carbamyl phosphate transcarbamylase and shown that the equilibrium of the reaction which it catalyzes strongly favors citrulline synthesis. The enzyme is specific for ornithine, and, of particular interest, appears to be identical with the citrulline phosphorylase of Krebs *et al.* (199). Thus, the reversal of Reaction *d* can be coupled with Reaction *e*, catalyzed by aspartate-carbamyl phosphate transcarbamylase (200, 201) from *E. coli* or various rat tissues, to effect the formation of carbamyl aspartate.



According to a recent report by Wu & Wilson (202), the role of aspartic acid in pyrimidine synthesis as studied in their own and other laboratories may now be formulated as follows:



It is evident, therefore, that the intermediates of the urea cycle contribute to pyrimidine synthesis only to the extent that they lead to the formation of carbamyl aspartic acid by the reactions described above.

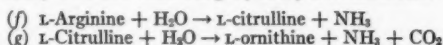
Since the evidence for a different pathway of urea synthesis via glutamine was examined critically a year ago by Kamin & Handler (203), it will suffice to note here that Bach & Smith (204) have reported further evidence which leads them to postulate a pathway other than the Krebs-Henseleit cycle. Burke & Miller (205) have proposed a similar pathway for the conversion of glutamine to urea in the livers of animals fed 3'-methyl-dimethylaminoazobenzene, a compound which appears to cause an impairment in urea synthesis. Clearly, studies with isolated enzyme systems are needed before the existence of such an alternate route for urea synthesis can be accepted.

Evidence that urea cannot be considered solely as an end product of metabolism is provided in the demonstration by Rose & Dekker (206) that urea nitrogen is utilized for the synthesis of nonessential amino acids when the latter are excluded from the food and no other nitrogen source is available. In their experiments,  $\text{N}^{15}$ -urea was administered to growing rats receiving a diet containing either 18 per cent casein or the essential amino acids at minimal levels. Extensive utilization of the urea, as judged by the appearance of  $\text{N}^{15}$  in the excreta and in individual amino acids of the carcass proteins (particularly cystine, glutamic acid, and aspartic acid), was observed only in the animals receiving the latter ration. Two studies which do not seem to be in complete accord with our current concepts of urea synthesis are the observation of Rust *et al.* (207) that after the injection of glucose- $\text{U}-\text{C}^{14}$  the urea formed is of higher specific activity than is the expired carbon dioxide, and the report of Salvatore & Saccone (208) that both L- and D-alanine lead to increased ammonium chloride excretion in rats, whereas only the L-isomer leads to increased urea excretion.

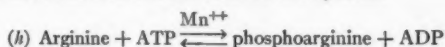
Greenstein and his associates (209 to 215) have made an extensive study of the metabolism of amino acids *in vivo* which has culminated in the demonstration, of medical significance, that arginine administration protects against lethal doses of ammonia. A specific mixture of the ten essential L-amino acids, administered intraperitoneally to rats, was found to be less

toxic than a similar mixture not containing arginine hydrochloride. Furthermore, L-arginine or citrulline administration 60 min. prior to the intraperitoneal injection of a lethal dose of ammonium acetate resulted in complete protection. Since liver slices prepared from the animals which had received either of these two protective compounds showed accelerated ammonia consumption and urea formation, the authors concluded that the protection was due to acceleration of the Krebs-Henseleit urea cycle. From a study of the effect of the route of administration of arginine on the protection against lethal doses of ammonia, they further concluded that this amino acid is most effective when brought to the liver as directly as possible (215).

*Other routes of ornithine, citrulline, and arginine metabolism.*—Yeast and many bacteria carry out the following hydrolytic reactions:

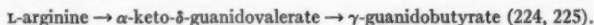


Petrack, Sullivan & Ratner (216) have partially purified arginine desiminase, which catalyzes Reaction *f* in *Streptococcus faecalis* extracts. The reaction proceeds to completion in the absence of phosphate and cannot be reversed even in the presence of ATP. A similar hydrolytic reaction converting canavanine to O-ureidohomoserine has been reported by Kihara & Snell (217); of particular interest, the enzyme catalyzing this reaction in *S. faecalis* appears to be identical with arginine desiminase. Morrison *et al.* (218), in extension of the work of others (219, 220), have obtained arginine phosphokinase (which catalyzes Reaction *h*) in highly purified form from sea crayfish tail muscle and have studied the kinetics of the system.



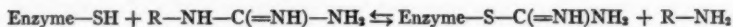
The kinase is specific for ATP but phosphorylates homoarginine and canavanine as well as arginine.

*Other amidine compounds.*—A new pathway of arginine metabolism of considerable interest has recently been discovered in Roche's laboratory (221, 222, 223). An adaptive enzyme system in *Streptomyces griseus* apparently oxidatively decarboxylates arginine to guanidobutyramide, which in turn is hydrolyzed with the formation of guanidobutyrate and ammonia. Of further interest, deguanidases in this organism act on various compounds, thus converting  $\gamma$ -guanidobutyrate to  $\gamma$ -aminobutyrate, for example. In mammalian tissues and in insects, however, the metabolic pathway is apparently as follows:



Fuld (226) and Walker (227) have suggested earlier than an enzyme-amidine complex may be involved in transamination reactions and Ratner & Rochovansky (228) have proposed that the amidine donor and acceptor are attached to the enzyme at sites adjacent to the catalytic area and that transamination occurs by a displacement reaction. Nakatsu (229) and Walker (230) have independently noted that a free sulfhydryl group is nec-

essary for transamidinase activity and have suggested that an enzyme-isothiourea derivative may function in biological guanido group-forming reactions in the following manner:



Walker has also described the previously unreported guanidinoacetate-glycine, arginine-hydroxylamine, and guanidinoacetate-hydroxylamine transamidinations and has identified the product formed with hydroxylamine as hydroxyguanidine.

*Arenicola marina* muscle has been reported to contain a specific kinase which catalyzes the conversion of taurocyamine to the corresponding phosphoamidine in the presence of ATP, and *Nereis diversicolor* has been found to contain a specific glycoxyamine kinase (231, 232). These phosphoamidines have been isolated from these sources (231, 232), and taurocyamine and carbamyl taurine have been identified in the urine of rats (233).

**Polyamines.**—Little was known of the origin of the polyamines, spermidine and spermine, which are widely distributed in natural materials, until the recent investigations of Tabor *et al.* (234, 235, 236) and of Greene (237, 238) established putrescine and methionine as precursors of the 4-carbon and 3-carbon chains, respectively. Adenosyl-L-methionine is now known to undergo enzymatic decarboxylation in *E. coli* extracts, presumably with the formation of adenosylthiomethylpropylamine, which then reacts with putrescine to furnish spermidine,  $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$ . Thiomethyladenosine would be expected as the other product of the reaction. Herbst *et al.* (239) have found that many Gram-negative bacteria contain putrescine and excrete "growth factor" activity into the medium, whereas most Gram-positive bacteria neither store nor excrete putrescine and polyamines. The role of the polyamines as growth factors has been emphasized by the report of Kihara & Snell (240) that spermine and spermidine are highly active as growth stimulants for *Lactobacillus casei*. Furthermore, the superior growth-promoting property of crude casein digests as compared to digests of purified casein made with recrystallized trypsin was found to be due to the presence of steam-volatile amines.

## HISTIDINE

**Histidine degradation.**—Several of the enzymes which participate in histidine breakdown in animal tissues and microorganisms have been purified and characterized. Tabor & Mehler (241) have demonstrated that histidase, purified from *P. fluorescens* or guinea pig liver, requires both pyrophosphate and a thiol for full activity. On the other hand, Koizumu *et al.* (242) have reported that this enzyme, purified from rabbit liver, requires the presence of cobaltous ions and folic acid as well as glutathione for maximal activity. Urocanic acid, the product of the histidase reaction, is further degraded to formiminoglutamic acid by the enzyme urocanase (243). Using a fourfold purified urocanase preparation from cat liver, Miller & Waelsch (244, 245) have investigated the mechanism of the reaction and have obtained evidence

for the accumulation of an oxidized intermediate in the presence of dichlorophenolindophenol. Although the compound has not been isolated, it has the properties expected of 5-imidazolone-4-acrylic acid. It is proposed that urocanase as a single enzyme catalyzes the formation of this intermediate, its reduction to 5-imidazolone-4-propionic acid, and hydrolysis of the latter compound to yield formiminoglutamic acid. The mechanism of conversion of formiminoglutamic acid to glutamic acid is discussed in the section on glycine and serine metabolism in this review.

Ichihara and his associates (246 to 249) have provided evidence for a pathway of urocanate degradation which involves the utilization of molecular oxygen with the apparent formation of hydantoin acrylic acid. It remains to be established, however, whether this transformation is related to the oxidative reaction described by Miller & Waelsch.

A third degradative pathway for histidine has been thoroughly investigated in Hayaishi's laboratory (250, 251). Imidazoleacetic acid in the presence of DPNH and molecular oxygen is converted to aspartic acid, ammonia, and formate by extracts of *Pseudomonas* sp. The individual enzymes in this pathway have been purified, and the following reactions have been clearly established:

- (a) Imidazoleacetic acid + DPNH + H<sup>+</sup> + O<sub>2</sub>  
→ DPN<sup>+</sup> + H<sub>2</sub>O + [imidazolone acetic acid] → formiminoaspartic acid
- (b) Formiminoaspartic acid + H<sub>2</sub>O → formylaspartic acid + NH<sub>3</sub>
- (c) Formylaspartic acid + H<sub>2</sub>O → aspartic acid + formic acid

**Histidine biosynthesis.**—Although Magasanik (252) and Neidle & Waelsch (253) have demonstrated that mutants of *E. coli* are capable of incorporating the N-1 and C-2 atoms of the guanine nucleus as a unit into the N-1 and C-2 positions of histidine, more recent evidence (254) supports the view that adenine is the actual donor of these atoms. To account for the accumulation of 5-amino-4-imidazolecarboxamide ribotide during histidine synthesis and for the observed isotope distribution in histidine derived from various labeled substrates, Moyed & Magasanik (254) have proposed the following scheme:

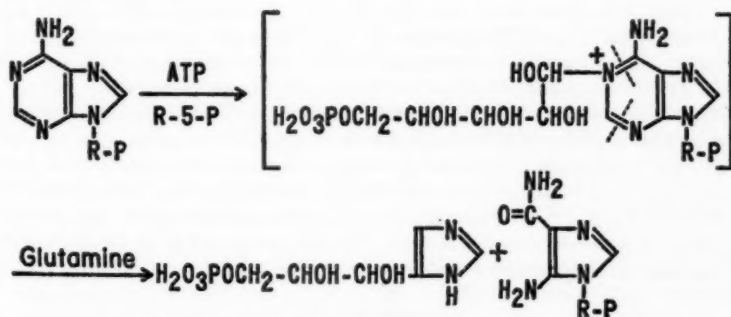


FIG. 2. Proposed reactions in histidine biosynthesis.



In continuation of his earlier thorough investigation of the pathway of histidine biogenesis in *Neurospora* mutants, Ames has recently provided detailed information on some of the individual enzymes involved in the conversion of imidazole glycerol phosphate to the amino acid. Imidazole glycerol phosphate dehydratase, which catalyzes the formation of imidazole acetol phosphate, requires the presence of manganese ions and a reducing agent such as mercaptoethanol (255). The action of a transaminase (256) then furnishes L-histidinol phosphate, which is hydrolyzed by a specific phosphatase (257). The conversion of histidinol to histidine was established earlier.

*Histidine derivatives.*—Following the demonstration that S<sup>35</sup>-sulfate is incorporated into ergothioneine by fungi but not by bacteria or animals (258, 259), Melville *et al.* (260) and Heath & Wildy (261) have found that in *N. crassa* and *Claviceps purpurea* histidine-2-C<sup>14</sup> is converted to ergothioneine-2-C<sup>14</sup> apparently without cleavage of the imidazole ring. Methionine can serve as a source of both the sulfur atom and the methyl groups, but as a sulfur donor cysteine is somewhat more effective than methionine. Heath & Wildy (262) have found that when the ergot fungus, *C. purpurea*, is grown in a medium containing acetate-2-C<sup>14</sup>, the resulting ergothioneine and histidine are labeled identically. This organism is unable to bring about the conversion of histamine to either histidine or ergothioneine (263), in accord with the known irreversibility of amino acid decarboxylations. Although ergothioneine has long been recognized as a constituent of mammalian erythrocytes, its metabolic significance is uncertain. Grossman & Kaplan (264) have recently observed that nicotinamide inhibition of nicotinamide ribosidase from bovine erythrocytes decreases as this enzyme is purified. Nicotinamide had no inhibitory effect on their purest enzyme preparation, but in the presence of ergothioneine the nicotinamide effect was restored; other sulphydryl compounds were not effective. Yanasugandha & Appleman (265) have found that an ergothioneine-metabolizing strain of *Alcaligenes faecalis* converts this compound to trimethylamine and thiourocnic acid. This reaction bears a striking resemblance to the deamination of histidine by histidase.

McManus (266) has found that the methyl group of methionine is approximately 20 times as effective as either formate or formaldehyde as a methyl donor for anserine in the rat. In contrast to the report of Williams & Krehl (267) that addition of  $\beta$ -alanine increased anserine and carnosine production in rat liver slices, Winnick & Winnick (268) failed to obtain significant incorporation of C<sup>14</sup>-labeled  $\beta$ -alanine into these dipeptides in rat and chicken liver slices.  $\beta$ -Alanine was found, however, to be incorporated into the dipeptides by strips of rat and chicken muscle. In view of the finding by Wood (269) that the hydrolytic enzyme carnosinase is present in most animal tissues but not in striated muscle, it is not yet possible to conclude that carnosine is synthesized exclusively in muscle.

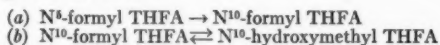
#### GLYCINE AND SERINE

*Role of folic acid derivatives in the glycine-serine interconversion.*—Work



in the past year on the serine-glycine transformation has focused on the nature of the  $C_1$  units involved. For a recent review of the role of the  $C_1$  unit in purine biosynthesis, readers are referred to a discussion by Greenberg & Jaenicke (270).

Investigations in several laboratories have led to the conclusion that the formyl group of the citrovorum factor ( $N^6$ -formyl THFA) undergoes transfer to  $N^{10}$  according to Reaction *a*, and that the resulting compound is then reduced according to Reaction *b*:



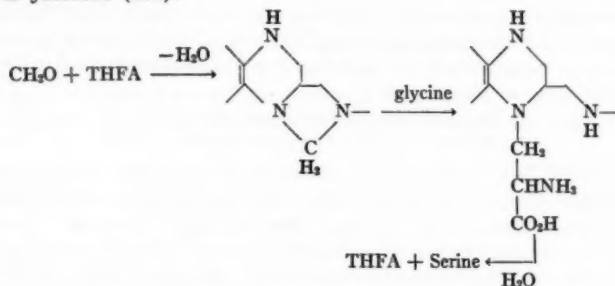
In further work on the metabolism of the hydroxyamino acids by sheep liver extracts, Peters & Greenberg (271) have confirmed the earlier observation (272) that Reaction *a* is ATP-dependent and have shown that two equivalents of orthophosphate are liberated in the conversion. Reaction *b* occurs in the presence of TPNH and is reversible (270, 271). Osborn & Talbert (273) have recently obtained partially purified preparations of this TPN-dependent dehydrogenase from beef and pigeon liver.

The enzymatic reduction of folic acid has recently been clarified. According to Wright & Anderson (274), extracts of *C. sticklandii* catalyze the reduction of this compound to dihydrofolic acid (DHFA) in the presence of CoA and a substrate such as pyruvate, serine,  $\alpha$ -ketobutyrate, or methionine. The function of these substrates most likely is to reduce pyridine nucleotides, for Futterman & Silverman (275, 276) have demonstrated a TPNH-dependent reduction of folic acid to tetrahydrofolic acid (THFA) and the reduction of DHFA to THFA in the presence of TPNH or DPNH in a chicken liver enzyme system. The structural requirements for the activity of the folic acid derivative in  $C_1$  transfer have been examined by Blakley (277, 278), who has observed that of a number of compounds tested, only DHFA, THFA,  $N^{10}$ -formyl THFA, and the tetrahydro derivatives of pteroyltriglutamic acid and pteroylheptaglutamic acid were active in serine synthesis in a liver preparation. The 2-amino and 4-hydroxy groups, the hydrogenated pyrazine ring, and one or more glutamic acid residues are essential for activity.

In support of earlier observations by Blakley (279) and Jaenicke (280), it has been shown that pyridoxal-5-phosphate is required for  $N^{10}$  formylation of THFA by L-serine (281, 282). The enzyme system catalyzing this reaction is specific for L-serine and requires the presence of manganous ions. In a study of the over-all conversion of serine to formate, Huennekens (283) has found a beef liver deacylating enzyme which hydrolyzes  $N^{10}$ -formyl THFA to formate and THFA.

Kisliuk (284) has concluded that  $N^{6,10}$ -methylene THFA may be the actual donor of the  $\beta$ -carbon of serine since hydrogen atoms on both the 5 and 10 positions of THFA are necessary for formaldehyde binding. He has also noted that THFA is much more effective than DHFA for the enzymatic synthesis of serine and has suggested that the apparent activity of the latter cofactor may be due to the presence of THFA as an impurity, or that a dis-

mutation reaction may occur between two molecules of DHFA to yield THFA and folic acid. The scheme as presented by Kisliuk for the biosynthesis of serine from formaldehyde and glycine is in accord with the observations of Jaenicke (280):



In an extension of earlier studies in Buchanan's laboratory on serine as a C<sub>1</sub> donor for purine biosynthesis, Warren (285) has described two trans-formylases from chicken liver which effect the over-all conversion of IMP and glycinamide ribotide to aminoimidazolecarboxamide ribotide and formylglycinamide ribotide, as follows:

- (c)  $\text{IMP} + \text{THFA} \rightleftharpoons \text{aminoimidazolecarboxamide ribotide} + \text{formyl THFA}$   
 (d)  $\text{Formyl THFA} + \text{glycinamide ribotide} \rightarrow \text{formylglycinamide ribotide} + \text{THFA}$

Although citrovorum factor *per se* has not generally been believed to be a direct formylating agent in mammalian tissues, Silverman *et al.* (286) have obtained a highly purified enzyme from pig liver which catalyzes the reversible transfer of the formyl group from citrovorum factor (but not from N<sup>10</sup>-formyl THFA) to glutamic acid with the formation of N-formylglutamic acid. Glutamine and isoglutamine also serve as acceptors for the formyl group, but at a slower rate. Miller & Waelsch (287) have also obtained evidence that formylglutamic acid formylates THFA in the N<sup>6</sup> position.

**Formimino group transfer.**—The role of folic acid derivatives in formimino group transfer, elucidated by Rabinowitz and his associates (288, 289), has also been the subject of more recent reports (287, 290 to 292). These reactions include transfer from formiminoglycine in *Clostridium cylindrosporum* and from formiminoglutamic acid (a histidine metabolite) in liver to yield N<sup>6</sup>-formimino THFA, which then undergoes the following reactions in both bacterial and liver preparations:

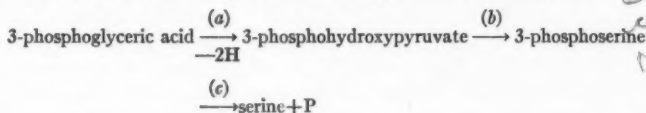
- (e)  $\text{N}^6\text{-formimino THFA} \rightarrow \text{N}^{5,10}\text{-methenyl THFA} + \text{NH}_3$   
 (f)  $\text{N}^{5,10}\text{-methenyl THFA} \xrightarrow{\text{H}_2\text{O}} \text{N}^{10}\text{-formyl THFA}$

Cyclodeaminase, which catalyzes Reaction e, has no action on N<sup>6</sup>-formyl THFA. Enzymatically formed N<sup>5,10</sup>-methenyl THFA undergoes hydration according to Reaction f in the presence of the enzyme cyclodehydrase. Evidence that folic acid is a necessary cofactor for the metabolism of formimino-

glutamic acid in human subjects has been provided by Broquist & Luhby (293, 294), who have noted that children excrete the latter compound in the urine when undergoing therapy with folic acid antagonists for acute leukemia.

The relationship of several other compounds to  $C_1$  metabolism has also been noted. The specific role of vitamin  $B_{12}$  in  $C_1$  metabolism has not yet been established. Stekol *et al.* (295) and Young *et al.* (296) have confirmed earlier reports that vitamin  $B_{12}$  is not involved in transmethylation reactions, and the latter investigators have obtained evidence that vitamin  $B_{12}$  stimulates the synthesis of methyl groups in rats *in vivo*. Tanaka (297) has observed that vitamin  $B_{12}$  markedly stimulates the conversion of folic acid to citrovorum factor by resting cell suspensions of *S. faecalis*. Furthermore, inhibition of this conversion by histamine, putrescine, or cadaverine is partially overcome by the addition of the vitamin. Doctor & Awapara (298), however, apparently could not detect any effect of vitamin  $B_{12}$  on the incorporation of formaldehyde into methionine by extracts of acetone powder from pigeon liver. Dinning *et al.* (299) have observed a lowered utilization of formate for serine and methionine biosynthesis by liver homogenates prepared from ascorbic acid-deficient guinea pigs; Herranen & Mueller (300) have found that the incubation of rat uterine tissue with estradiol-17 $\beta$  causes a stimulation in the incorporation of the  $\beta$ -carbon of serine into purines.

*Other metabolic reactions of glycine and serine.*—In addition to the biosynthetic route from glycine and the  $C_1$ -unit, serine is known to be formed from 3-carbon compounds derived from carbohydrates. By means of experiments with glycerol-1,3- $C^{14}$ , Koeppe *et al.* (301) have found that, in the intact rat, serine is formed from a 3-carbon intermediate of glycolysis other than pyruvate. Ichihara & Greenberg (302) report that a crude enzyme system from rat liver can catalyze the following transformations:



3-Phosphohydroxypyruvate is formed by dehydrogenation of 3-phosphoglycerate (Reaction a). Glutamate is the most effective amino donor in the transamination reaction (Step b), but several other amino acids are also active. Sallach (303) has found, on the other hand, that alanine is the only effective donor in the transamination of hydroxypyruvate to form serine in dog liver preparations. Finally, serine is generated by the action of a phosphatase (Reaction c).

Although there are several pathways for the metabolism of L-serine, Elwyn *et al.* (304) have obtained indirect evidence that D-serine is degraded slowly by a single pathway which leads to the formation of carbohydrate intermediates. Schlossman & Lynen (305) have reported a new synthetic

}  
can get  
carbohydrate  
from serine  
in vivo

pathway for the formation of cysteine in yeast by the reaction of serine and hydrogen sulfide.

In a study of the biosynthesis of sphingosine by the soluble fraction of rat brain, Brady *et al.* (306, 307) have observed that radioactivity from serine-3-C<sup>14</sup> appears in positions 1 and 2 of sphingosine. The evidence indicates that this isotope incorporation does not represent indirect incorporation of radioactivity from one- or two-carbon compounds. The conversion of serine to sphingosine requires pyridoxal-5-phosphate, magnesium ions, TPN, DPN, and an unidentified substance from an extract of boiled rat liver.

In a further study of the nature of phosphate esterification, coupled to glycine reduction in *C. sticklandii* (308), Stadtman (309) has resolved the enzyme system into two components. The intermediates in the over-all reaction,  $\text{glycine} + \text{R(SH)}_2 + \text{ADP} + \text{P} \rightarrow \text{acetate} + \text{NH}_3 + \text{RS-SR} + \text{ATP}$ , have not been identified. Bachrach (310) has obtained evidence that in *P. aeruginosa* glycine is converted into glyoxylic acid by transamination. The glyoxylic acid is further metabolized entirely by the isocitritase pathway.

Shemin has proposed earlier that in the initial step of porphyrin biosynthesis an active form of succinate, such as succinyl CoA, condenses with glycine to form  $\alpha$ -amino- $\beta$ -ketoadipic acid, which then decarboxylates to give  $\delta$ -aminolevulinic acid. The conversion of this product to protoporphyrin has recently been described by Schiffman & Shemin (311). Indirect evidence that CoA and pyridoxal-5-phosphate are involved in this condensation has been obtained in two laboratories. Schulman & Richert (312) have found that the synthesis of heme from glycine and succinate is reduced in the red blood cells of ducklings with a dietary pantothenic acid or pyridoxine deficiency, but that heme synthesis from  $\delta$ -aminolevulinic acid remains normal under these conditions. In pyridoxine deficiency, the addition of pyridoxal-5-phosphate to the cells, *in vitro*, stimulates the formation of heme from glycine and succinate. Pantothenate and CoA are ineffective in restoring normal porphyrinogenesis in cells from pantothenate-deficient birds, but the injection of this vitamin into the birds before the blood is drawn restores the glycine incorporation to normal. Similar results were obtained by Lascelles (313) in a study of the effect of vitamins on porphyrin synthesis by cell suspensions of *Tetrahymena vorax*. Evidence was also obtained that riboflavin or one of its derivatives may be involved in the conversion of  $\delta$ -aminolevulinic acid to porphyrin.  $\alpha$ -Amino- $\beta$ -ketoadipic acid is an unstable compound which decarboxylates spontaneously to yield  $\delta$ -aminolevulinic acid, but the diethyl ester is stable. Weliky & Shemin (314) have administered the ester labeled in carbon 2 with C<sup>14</sup> to rats and have found that the labeled carbon appears in porphyrin and in urinary hippuric acid. The ester is readily hydrolyzed by an esterase in rat liver.

It is generally assumed that choline is synthesized by the stepwise addition of methyl groups to ethanolamine, but a direct demonstration of the separate stages of this transformation is still wanting. Pilgeram *et al.* (315)

observed the incorporation of the methyl group of methionine into the choline moiety of phosphatidyl choline by rat liver slices, but all attempts to show the same reaction in rat liver homogenates were unsuccessful. In studying the formation of monomethylethanolamine by mutants of *N. crassa*, Nyc (316) has found that formate is a much better precursor of the methyl group than is methionine. Ahmad & Jabar (317) have reported the synthesis of choline from formate and ethanolamine by the chick pea.

The choline dehydrogenase of liver mitochondria has been studied in several laboratories (318, 319, 320). This enzyme has a pH optimum of about 7.4 and is inhibited by hydroxylamine, ammonium salts, and high concentrations of choline. Byerrum *et al.* (321) have obtained evidence suggesting the presence of a choline oxidase system in tobacco plants, and Artom *et al.* (322, 323) have reported an enzyme present in rat liver mitochondria which converts choline to an unidentified intermediate. Knowles & Sakami (324) have found that a digitonin extract of rat liver mitochondria oxidizes sarcosine to formaldehyde and glycine.

#### THREONINE AND HOMOSERINE

In continuation of their earlier studies, Umbarger & Brown (325) have obtained evidence that *E. coli* extracts contain two distinct L-threonine deaminases. One of the deaminases, shown earlier by Wood & Gunsalus (326) to require pyridoxal-5-phosphate, AMP, and glutathione, was found to be an adaptive enzyme. The second deaminase, which is present in extracts of wild type *E. coli* but absent from mutants which are unable to convert threonine to  $\alpha$ -ketobutyrate as a step in isoleucine synthesis, requires only pyridoxal-5-phosphate and is inhibited by isoleucine. Kinetic studies have led to the proposal that the Wood-Gunsalus deaminase combines with one molecule of substrate, whereas the other enzyme combines with two. Both of these deaminases act on serine, and evidence is presented that *E. coli* cells contain a third deaminase which is serine-specific. Walker (327) has purified the L-threonine deaminase of the rumen microorganism LC-1; pyridoxal-5-phosphate, the cofactor, can be reversibly dissociated from the enzyme by lyophilization in the presence of glutathione and ammonium sulfate.

A complex reaction which includes deamination has been studied in detail by Matsuo & Greenberg (328), who have isolated in crystalline form a yellow enzyme which converts either homoserine or cystathionine to  $\alpha$ -ketobutyrate. On the basis of absorption spectra and other physical and chemical properties, pyridoxal-5-phosphate is believed to be bound to the enzyme. The optimal pH values and the rates of formation of  $\alpha$ -ketobutyrate are of the same magnitude for the two substrates. It appears that threonine may be decarboxylated by *S. griseus*, for Krasna *et al.* (329) have shown that threonine is converted to the 1-amino-2-propanol residue of vitamin B<sub>12</sub> in this organism.

Karasek & Greenberg (330) have been able to demonstrate that the thre-

onine and allothreonine aldolase activities of sheep liver are due to different enzymes. In each case the products are acetaldehyde and glycine, and the reactions are readily reversible. A metal ion requirement could not be demonstrated, but pyridoxal-5-phosphate was required for maximal activity. In a study of the acetaldehyde-glycine condensation in rat liver preparations, Gilbert (331) has suggested that this reaction is of minor importance in mammals because of the lack of acetaldehyde, but that it may serve a major role in organisms such as yeast.

The enzymatic reactions established by Black & Wright (332) for the biosynthesis of threonine from aspartate in microorganisms apparently also occur in higher plants (333). In a study of the conversion of homoserine to threonine, which is the final step in this pathway, Watanabe and his associates (334, 335) have isolated a new intermediate, O-phosphohomoserine. This product was formed from L-homoserine and ATP in the presence of magnesium ions and a partially purified preparation of yeast homoserine kinase. A second yeast enzyme converted the phosphorylated compound to threonine.

#### LYSINE

As summarized in a recent review by Work (336), lysine arises from  $\alpha$ -amino adipic acid in *Neurospora* and yeast, but by a different pathway from  $\alpha,\epsilon$ -diaminopimelic acid (DAP) in bacteria. The latter metabolite is present not only in the soluble amino acid fraction of certain bacteria, but also in the insoluble residues, apparently as a cell wall constituent. Gilvarg (337) has obtained a DAP-requiring *E. coli* mutant which accumulates a compound nutritionally active for this same auxotroph only after acid hydrolysis. This compound, which has been identified as N-succinyl-L-diaminopimelic acid, is hydrolyzed by extracts from wild type *E. coli*, presumably with the formation of L-diaminopimelic acid. On the other hand, as expected, extracts of the mutant which makes the succinyl derivative do not contain this hydrolase. The following scheme indicates our present knowledge of this biosynthetic pathway:



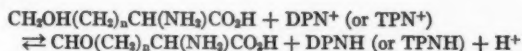
Since the substrate of diaminopimelic decarboxylase is the *meso* form, the formation of this species from the L isomer needs to be accounted for. Antia, Hoare & Work (338) have recently described the occurrence of a bacterial racemase which catalyzes the interconversion of these isomers. The racemase, like the decarboxylase, acts at the site of the D configuration in *meso*-diaminopimelic acid, but has no effect upon the D isomer. The racemase is even more widely distributed among bacteria than is the decarboxylase. A survey of the distribution of diaminopimelic acid in bacteria has revealed considerable variation in the occurrence of the L and *meso* isomers, with the D form generally absent (339).

The relation of diaminopimelic acid metabolism and sporulation in *Bacillus sphaericus* has been examined by Powell & Strange (340). In this organism, as in other bacilli (341), pyridine-2,6-dicarboxylic acid (dipi-



colinic acid), a possible metabolite of diaminopimelic acid, is deposited as a calcium salt in the developing spore. Of particular interest, a "spore peptide" containing hexosamines, diaminopimelic acid, aspartic acid, glutamic acid, and alanine appears in spore extracts and germination exudates. The latter two amino acids are known to be present as the D isomers in the spore peptide in *Bacillus megatherium* (342). Peptides of similar constitution appear to be widely distributed in bacterial cell walls (343, 344, 345, 346). Meadow *et al.* (347) have reported that mutants requiring diaminopimelic acid lyse during the logarithmic growth phase when grown in the presence of lysine and sub-optimal concentrations of the diamino acid. Their experiments may indicate a failure in cell wall synthesis because of diaminopimelic acid deficiency, since electron micrographs after lysis showed cell envelopes which had lost the protoplasmic contents.

The previously considered role of  $\alpha$ -amino adipic- $\delta$ -semialdehyde, as an intermediate in the conversion of  $\alpha$ -amino adipic acid to lysine in *Neurospora* (348), is given indirect support by recent findings of Yura & Vogel (349), who have shown that *Neurospora* extracts catalyze the oxidation of  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid, a lysine precursor in this organism, to the aldehyde acid as follows:



This  $\omega$ -hydroxy- $\alpha$ -amino dehydrogenase acts on substrates where  $n = 1$  to 3, and appears to be specific for the L isomers. The relationship of this enzyme to yeast homoserine dehydrogenase (350) appears to be uncertain.

New information on lysine degradation has been provided by Meister & Buckley (351), who have briefly reported the enzymatic reduction of  $\Delta^1$ -piperidine-2-carboxylic acid (which is in equilibrium with  $\alpha$ -keto- $\epsilon$ -amino adipic acid) in the presence of DPNH or TPNH. The partially purified enzyme from rat liver which catalyzes the generation of L-pipecolic acid in this manner also effects the reduction of  $\Delta^1$ -pyrroline-5-carboxylic acid to proline, and may therefore be identical with or similar to enzymes in *Neurospora* (352) and liver (353) described previously. In agreement with findings in other organisms, pipecolic acid appears not to be a precursor of lysine in *Ustilago maydis* (354).

Paik *et al.* (355) have purified an  $\epsilon$ -lysine acylase from pig kidney. The enzyme is specific for the L-configuration of various  $\epsilon$ -acylated lysine derivatives, but its metabolic function is not yet clear. Kamahora (356) has reported the occurrence of  $\delta$ -aminovaleric acid as a product of L-lysine metabolism in a strain of *Streptomyces* adapted for the latter amino acid. This oxidative transformation appears to be the same, in principle, as the formation of guanidobutyric acid from arginine, as discussed elsewhere in this review.

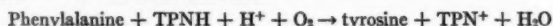
#### PHENYLALANINE AND TYROSINE

*Phenylalanine and tyrosine biosynthesis.*—The pathway by which carbohydrates are converted to phenylalanine and tyrosine has recently been re-



viewed in detail by Davis (357). Despite the excellent utilization of sedoheptulose-1,7-diphosphate for shikimic acid synthesis in *E. coli*, isotopic experiments now indicate that this phosphorylated sugar is not an obligatory intermediate in aromatic biosynthesis (358, 359). Rather, shikimic acid appears to be formed more directly from tetrose phosphate and triose phosphate units. Although several lines of evidence indicate that quinic acid is not convertible to shikimic acid in *E. coli* (360), Carr *et al.* (361) have found that cell suspensions of *Lactobacillus pastorianus* var. *quinicus* catalyze the interconversion of these two acids. Prephenic acid, which is known as a precursor of aromatic amino acids in *E. coli*, has recently been isolated from the growth medium of a tryptophan-phenylalanine-tyrosine-requiring mutant of *N. crassa* (362). Miller & Simmonds (363) have provided evidence that in *E. coli* the conversions of prephenic acid to tyrosine and to phenylalanine are irreversible.

*Degradation of phenylalanine and tyrosine.*—In a careful investigation of the reaction by which phenylalanine is converted to tyrosine in animal tissues, Kaufman (364, 365, 366) has partially purified the two protein fractions which had previously been shown by Mitoma (367) to be necessary for this conversion. One of the protein fractions appears to catalyze the reduction of an unidentified cofactor by TPNH. Balance studies support the following formulation of the over-all reaction:



Wallace *et al.* (368) have found that a homogenate of a liver biopsy specimen obtained from a patient with phenylpyruvic oligophrenia failed to catalyze the conversion of phenylalanine to tyrosine. The addition of one of Mitoma's rat liver fractions to the inactive phenylketonuric liver homogenate resulted in striking activation of phenylalanine hydroxylation. The role of each of the two enzymes in phenylalanine oxidation must be determined before the defect in this disease can be pinpointed.

The products of phenylalanine metabolism excreted by phenylketonuric patients appear to inhibit reactions in tyrosine breakdown. Thus, Bickis *et al.* (369) have found that phenylpyruvic acid exercises a considerable inhibitory action on the conversion of tyrosine to acetoacetate in liver slices and homogenates, and Fellman (370) has observed the inhibition of 3,4-dihydroxyphenylalanine (DOPA) decarboxylase by phenylpyruvic, phenyllactic, and phenylacetic acids.

With the aid of an assay based on the absorption of ultraviolet light by enol borate complexes, Knox & Pitt (371) have purified an enzyme from pig kidney which catalyzes the keto-enol tautomerization of phenylpyruvate, *p*-hydroxyphenylpyruvate, and *m*-hydroxyphenylpyruvate; the metabolic significance of this interesting reaction is not known. Hager *et al.* (372) have obtained evidence that the oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid is catalyzed by a single liver enzyme; oxygen is required, but a peroxidative reaction does not appear to be involved. The presence of glutathione and ascorbic acid is necessary to prevent inactivation of the enzyme during the reaction.

Several additional routes for phenylalanine and tyrosine degradation are now recognized. Lupine mitochondria oxidize phenylpyruvate with the production of benzaldehyde and carbon dioxide, but the intermediates in this reaction have not been identified (373). SenteShanmuganathan (374) has obtained evidence that tyrosine is converted to tyrosol in yeast by deamination, decarboxylation, and reduction of the resulting aldehyde by DPNH. *m*-Hydroxyphenylhydracrylic acid, which presumably originates from phenylalanine, has now been identified as a major phenolic constituent of human urine (375).

Nord and his associates (376, 377, 378) have obtained evidence that the pathway of lignin synthesis is as follows: carbohydrate  $\rightarrow$  shikimic acid  $\rightarrow$  *p*-hydroxyphenylpyruvic acid  $\rightarrow$  [intermediate]  $\rightarrow$  lignin. It appears that the intact aromatic ring of *p*-hydroxyphenylpyruvic acid is utilized for the formation of lignin, and that the side chain serves as the connecting link between the rings of the polymer. The methoxyl groups of lignin are derived from the methyl group precursors, serine, glycine, and formaldehyde (379).

*Metabolism of epinephrine and thyroxine.*—Kertesz & Zito (380) have improved the method of purification of tyrosinase from mushrooms (381). As an indication of the broad specificity of tyrosinase, Yasunobu & Daneliker (382) have found that this enzyme is able to effect the oxidation of the tyrosine residues of highly purified  $\alpha$ -lactoglobulin and ribonuclease. In general accord with the work of others, Goodall & Kirshner (383, 384, 385) have obtained evidence that the pathway of epinephrine formation is: tyrosine  $\rightarrow$  DOPA  $\rightarrow$  hydroxytyramine  $\rightarrow$  norepinephrine  $\rightarrow$  epinephrine. An adrenal particulate fraction converts hydroxytyramine to norepinephrine, and a soluble enzyme methylates the latter compound in the presence of S-adenosylmethionine. Following the observation by Armstrong and his associates (386, 387, 388) that 3-methoxy-4-hydroxymandelic and homovanillic acids are major urinary metabolites of epinephrine, Axelrod (389) has partially purified an enzyme from liver which, in the presence of S-adenosylmethionine, catalyzes methylation at position 3 of epinephrine, norepinephrine, hydroxytyramine, and 3,4-dihydroxybenzoic acid.

In continuation of their work on thyroxine, Roche *et al.* (390) have found that rat thyroid gland cultured *in vitro* retains the ability to synthesize thyroid hormones. The addition of anterior pituitary tissue intensely stimulated the synthesis of these hormones. Hartmann (391, 392) has purified a pig liver deiodase which attacks diiodotyrosine and was stimulated tenfold by the addition of pyruvate. In addition to the well-known action of thyroxine in uncoupling oxidative phosphorylation, this hormone has recently been found to inhibit malic dehydrogenase (393), transhydrogenase (394), acetyl phosphatase (395), and enzymes requiring pyridoxal-5-phosphate (396, 397).

#### TRYPTOPHAN

*Nicotinic acid synthesis.*—The sequence of reactions leading from tryptophan to nicotinic acid (tryptophan  $\rightarrow$  formylkynurenine  $\rightarrow$  kynurenine  $\rightarrow$  3-hydroxykynurenine  $\rightarrow$  3-hydroxyanthranilic acid  $\rightarrow$  nicotinic acid) is well

established. Recent studies have added more detailed information concerning this pathway. Civen & Knox (398) and Price & Dietrich (399) have obtained evidence that the adaptive formation of tryptophan peroxidase, which catalyzes Reaction *a*, represents a net synthesis of the enzyme. Additional support for the role of kynurenine as a nicotinic acid precursor has been obtained by Henderson *et al.* (400), who have found that both kynurenine and 3-hydroxykynurenine will support the growth of nicotinic acid-deficient rats. The hydroxylation step (Reaction *c*) has been shown by de Castro *et al.* (401) to occur in mitochondria in the presence of TPNH and oxygen, and Saito *et al.* (402) have demonstrated with a partially purified enzyme preparation that atmospheric oxygen is the source of the oxygen atom in the hydroxyl group.

The metabolic reactions of 3-hydroxyanthranilic acid are summarized in Figure 3. By incubating a mammalian liver preparation with 3-hydroxyanthranilic acid at 0°, Wiss *et al.* (403, 404) have been able to generate the aldehyde intermediate shown in brackets. This compound has been isolated and characterized by the preparation of derivatives. Although nicotinic acid

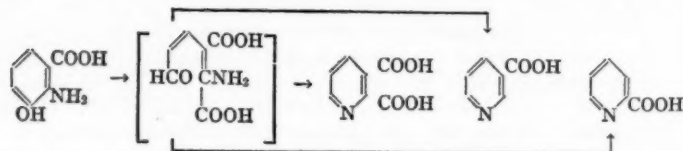


FIG. 3. Conversion of 3-hydroxyanthranilic acid to pyridine derivatives.

formation from the aldehyde is generally believed to occur directly by decarboxylation and subsequent ring closure, Hanks & Segel (405) have reported that quinolinic acid, which apparently is formed spontaneously from the aldehyde, may undergo decarboxylation to yield nicotinic acid. Since a liver enzyme which is able to convert 3-hydroxyanthranilic acid to picolinic acid cannot decarboxylate quinolinic acid, Mehler (406) has postulated that the aldehyde is decarboxylated before ring closure to form picolinic acid. When  $C^{14}$  labeled picolinic acid is administered to rats it is excreted quantitatively in the urine as its glycine conjugate (407). Suhadolnik *et al.* (408) have reported in a preliminary communication that picolinic acid is the major product formed from 3-hydroxyanthranilic acid by cat liver extracts and that quinolinic acid is the major product formed by rat liver extracts. Evidence has been obtained by Mehler & May (409) that a large part of the 3-hydroxyanthranilic acid which disappears cannot be accounted for by the known reactions involving the pyridine carboxylic acids.

*Metabolites of kynurenine and 3-hydroxykynurenine.*—Kynurenine transaminase has been partially purified from rat kidney (410) and from *Neurospora* (411). The keto acid corresponding to kynurenine has not been isolated, probably because it may be spontaneously and rapidly converted to kynurenic acid. Kynurenic acid and metabolically related compounds have been reported to produce a hypoglycemic response when injected into rats,

possibly by inhibition of insulinase (412). After ingestion of kynurenic acid, both human subjects and rats excrete quinaldic acid in the urine (413). The enzyme kynureninase, which catalyzes the conversion of kynurenine to anthranilic acid, has been obtained in an electrophoretically homogeneous state from pig liver by Wiss & Weber (414). Pyridoxal was found to be present in a hydrolysate of the enzyme preparation. By employing hydroxylamine to inhibit the pyridoxal phosphate-dependent decarboxylation of anthranilic acid in *E. coli*, McCullough (415) has been able to demonstrate a hydroxylation reaction leading to 3-hydroxyanthranilic acid. This hydroxylation step is dependent on the presence of an unidentified compound which is produced by preincubating the cells with *p*-aminobenzoic acid.

The metabolic reactions of 3-hydroxykynurenine are analogous to those of kynurenine. The former compound is converted to the quinoline derivative, xanthurenic acid, by a *Neurospora* transaminase (411) and by a partially purified preparation of kynurenine transaminase from rat kidney (415a). As noted above, 3-hydroxykynurenine undergoes loss of the side chain to give 3-hydroxyanthranilic acid. Although Rothstein & Greenberg (416) have observed that the administration of xanthurenic acid to rats results in the quantitative excretion by the kidney of this compound and its conjugates, it is now known that man and the rat are able to remove the 4-hydroxyl group to form the urinary metabolite, 8-hydroxyquinaldic acid (417). The 8-methyl ether of xanthurenic acid has also been identified in normal human urine (418).

*Alternate pathways of tryptophan degradation.*—For a number of years it has been known that certain aerobic microorganisms are able to carry out the sequence of reactions: anthranilic acid  $\rightarrow$  catechol  $\rightarrow$  muconic acid  $\rightarrow$   $\beta$ -ketoadipic acid. Katagiri & Hayaishi (419) have now obtained an enzyme system from a species of *Pseudomonas* which metabolizes  $\beta$ -ketoadipic acid by a series of reactions analogous to the degradation of acetoacetate by mammalian heart enzymes:

- (a)  $\beta$ -Ketoadipic acid + succinyl CoA  $\rightleftharpoons$   $\beta$ -ketoadipyl CoA + succinic acid
- (b)  $\beta$ -Ketoadipyl CoA + CoA  $\rightleftharpoons$  succinyl CoA + acetyl CoA

However, mammalian succinyl CoA transferase and  $\beta$ -ketothiolase do not catalyze these reactions.

Although the mechanism by which indoleacetic acid is formed from tryptophan is obscure (420), recent studies have clarified the metabolic fate of this compound in plants and fungi. Stutz (421) has obtained a homogeneous preparation of indoleacetic acid oxidase from *Lupinus albus* which requires the presence of a phenolic activator and manganous ions for maximal activity. The carboxyl group of indoleacetic acid is lost as carbon dioxide during the enzymatic reaction with the consumption of an equimolar amount of oxygen. A similar enzyme is present in wheat leaf extracts (422). Ray & Thimann (423, 424) have carefully studied the substrate specificity of the indoleacetic acid oxidase of the fungus *Omphalia flavidia* and have shown that the reaction probably occurs in at least two steps. Carbon dioxide and an unstable compound containing the indole group are formed, and the latter com-

pound is then rapidly oxidized nonenzymatically to form several unidentified compounds, one of which may be 3-methyldioxindole.

A physiologically important but quantitatively minor pathway of tryptophan degradation in animal tissues is the conversion of tryptophan to serotonin (5-hydroxytryptamine). The probable site of 5-hydroxytryptophan formation is the argentaffin cells (425, 426), since patients with argentaffinoma excrete large amounts of serotonin and 5-hydroxyindoleacetic acid in the urine. The hydroxylation of tryptophan appears to be irreversible, since 5-hydroxytryptophan cannot replace this "essential" amino acid in the nutrition of the rat (427). The indole ring of 5-hydroxytryptophan, unlike that of tryptophan, appears not to undergo metabolic cleavage (428), but the former compound is readily decarboxylated by a pyridoxal-dependent enzyme in kidney to yield serotonin (429, 430, 431).

**Biosynthesis of tryptophan.**—Data obtained by Yanofsky (432, 433) from nutritional, enzymatic, and genetic investigations give strong support to the mechanism proposed in Figure 4 for tryptophan biosynthesis in *E. coli*.

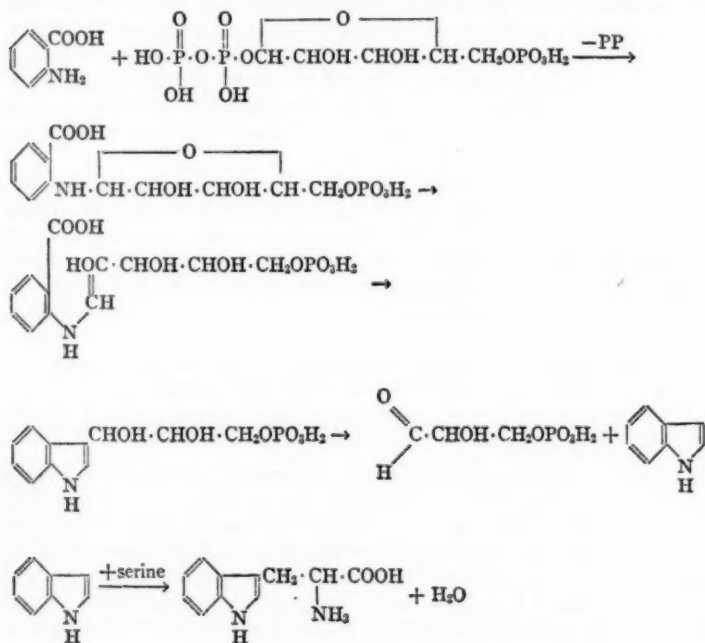


FIG. 4. Proposed scheme for tryptophan formation.

Studies with tryptophan-requiring mutants of *S. typhimurium* (434, 435) and *N. crassa* (436) indicate that these organisms synthesize tryptophan by

the same pathway. On the other hand, Parks & Douglas (437) have found that a tryptophan auxotroph of *Saccharomyces* accumulates a compound which has tentatively been identified as N-fructosyl anthranilic acid, and they postulate the occurrence of ring closure between C-1 of anthranilic acid and C-1 of fructose to yield an indole tetrose.

#### TRANSAMINATION AND RELATED REACTIONS INVOLVING PYRIDOXAL PHOSPHATE

Enumeration of the many contributions to our knowledge of the specificity of amino acid transaminases and decarboxylases and of their distribution in a variety of living organisms is beyond the scope of this review. Since an ever-increasing number of these enzymes are known to employ pyridoxal-5-phosphate as a coenzyme, the present discussion will be limited to the mechanism and control of reactions in which pyridoxine and its derivatives are involved.

Longenecker & Snell (438) have studied the interesting problem of the optical specificity of nonenzymatic transamination reactions and have shown that L-glutamate is formed preferentially when L-alanine or L-phenylalanine undergoes nonenzymatic transamination with  $\alpha$ -ketoglutarate in the presence of pyridoxal and metal ions. Similarly, D-glutamate predominates to a small extent when D-alanine or D-phenylalanine is employed. These findings indicate the participation of an asymmetric chelate. An intermediate chelate could be rendered asymmetric by the presence of a second amino acid residue in addition to the one which is passing to the imino form; in enzymatic transaminations the protein could occupy these positions if metal ions do indeed participate in these reactions. The same investigators (439) have compared the catalytic activities of 17 different metal ions in promoting several nonenzymatic pyridoxal-catalyzed reactions and have found that these activities generally parallel the magnitude of the stability constants of the chelates which the metals form with the relevant Schiff bases. This relationship and the reported isolation of the proposed intermediates (440) support the view that chelation is required for catalysis (441). The stabilities of the metal chelates of pyridoxamine have also been examined (442).

The observation that  $\alpha$ -methylserine and  $\alpha$ -methylolserine undergo aldol cleavage in the presence of pyridoxal and  $\text{Cu}^{++}$  or  $\text{Al}^{+++}$  ions indicates that an  $\alpha$ -hydrogen is not required for such reactions, which are analogous to the enzymatic cleavage of serine and threonine (443). Longenecker & Snell (444) have also noted that esters of serine and threonine are similarly degraded nonenzymatically:

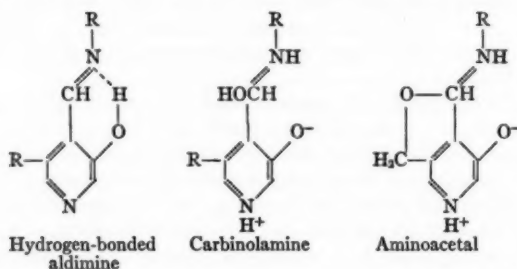


Furthermore, the chelation hypothesis (441) is supported by a recent study of the mechanism of the enzymatic decarboxylation of amino acids by Rothberg & Steinberg (445). By use of  $\text{H}_2\text{O}^{18}$ , these workers established that the carboxyl group is removed intact; they concluded that the linkage of the



carboxyl group to the enzyme is electrostatic or perhaps in the form of an ester, but that an acyl intermediate is ruled out.

Metzler (446) has shown by spectrophotometric titrations that the predominant Schiff base formed by pyridoxal and valine probably has a hydrogen ion bonded both to the phenolic oxygen and the imine nitrogen, thereby forming a resonating ring system with an absorption maximum at about 415  $m\mu$ . The hydrogen-bonding prevents the structure from assuming the zwitterion structure usual for 3-hydroxypyridines. Besides the yellow imine, amino acids form small amounts of a Schiff base absorbing at 330  $m\mu$ , taken to be the non-hydrogen-bonded carbinolamine, which would be zwitterionic and would probably be in an aminoacetal form:



Metzler also has provided formation constants for a number of pyridoxylidene derivatives of amines and amino acids. He has concluded that imine formation and breakdown occur rapidly and that the rate-limiting step in the nonenzymatic degradation of amino acids must be the tautomeric rearrangement of the imine.

Matsuo (447) has shown spectrophotometrically that pyridoxal phosphate forms with amines and amino acids Schiff bases which absorb at 278 and 415  $m\mu$  and that these bases also form metal chelates. In absolute alcohol transamination requires neither an elevated temperature nor a metallic ion (448).

According to a preliminary report by Jenkins & Sizer (449), the glutamic-aspartic transaminase of pig heart has been purified and obtained about 70 per cent pure. The presence of firmly bound pyridoxal phosphate has been confirmed; however, spectral study at various pH values suggests that the aldehyde group is not free but is presumably joined in imine linkage to an amino group of the enzyme. The absorption maximum is 363  $m\mu$ , however, thus indicating that the aldimine is not hydrogen-bonded, in contrast to the structure of model compounds as found by Metzler. Acidification to pH 4.8 produces a yellow color, a hydrogen ion presumably entering the hydrogen-bonding position. The absorption at pH 8.5 resembles that of the crystalline cystathionase-homoserine dehydrase of Matsuo & Greenberg (450). The aldehyde group of the glutamic-aspartic transaminase preparation could,



however, react with various agents, such as hydroxylamine, to leave the pyridoxal phosphate derivative still bound to the protein (449).

The question of the structures of these Schiff bases is intensified by a report by Christensen *et al.* (451) that certain peptides and proteins react with pyridoxal phosphate (and also with pyridoxal) to form the yellow Schiff bases, but that these then pass over almost completely to colorless imines absorbing at 330 to 335  $m\mu$ . By analogy, these should be carbinolamines, but it is not clear why pyridoxal phosphate should react to yield this form much more strongly than does pyridoxal, when stabilization of the carbinolamine linkage by aminoacetal formation is not possible with pyridoxal phosphate. Like the internal imine of glutamic-aspartic transaminase, the colorless imines of leucylglycylglycine and serum albumin with pyridoxal phosphate become yellow upon acidification, hydrogen-bonding presumably being established.

Three forms have so far been proposed for imines of pyridoxal phosphate: (a) the hydrogen-bonded aldimine, readily formed by simple amines and amino acids, and shown both for pyridoxal (446) and pyridoxal phosphate (452); (b) the non-hydrogen-bonded aldimine, as detected in glutamic-aspartic transaminase (449); and (c) the non-hydrogen-bonded carbinolamine, formed predominantly by amino acid esters, amides, and peptides (451). A fourth possible form, the hydrogen-bonded carbinolamine, may well explain the 278  $m\mu$  band also arising with amino acids (452).

In continuation of earlier studies on the participation of pyridoxal derivatives in amino acid transport, Christensen (453) has prepared numerous metal chelates of pyridoxylidene amino acids, most of which have the structure, metal-(pyridoxal-amino acid)<sub>2</sub>. When  $Fe^{+++}$  or  $Cu^{++}$  are employed, the metal is presumably linked to the phenolic oxygen, but this link appears to be absent in  $Mn^{++}$  or  $Ni^{++}$  chelates. As anticipated, infrared spectra showed a neutral structure for the copper chelate but a zwitterionic structure for  $Mn^{++}$  and  $Ni^{++}$  chelates. The substantial catalytic activity of the latter chelates was taken to mean that chelation to the phenolic oxygen is probably not necessary for catalytic activity. This implies that to be catalytic the metal does not need to maintain planarity in the Schiff base molecule.

Riggs *et al.* (454) have administered  $C^{14}$ -labeled  $\alpha$ -aminoisobutyric acid, a nonmetabolizable compound, to rats and have shown that pyridoxine-deficient animals have a lower concentration of this amino acid in the tissues (except liver) and a higher concentration in the serum than do normal animals. This effect could be overcome by the simultaneous administration of the vitamin and the amino acid to the deficient animals. Noall *et al.* (455) have found that administration of hydrocortisone to the normal rat promptly enhanced hepatic capture of the  $\alpha$ -aminoisobutyric acid. They attribute the catabolic effects of the hydrocortisone and related steroids to the accelerated amino acid breakdown (accompanied by accelerated gluconeogenesis and serum albumin synthesis) which follows the intensified amino acid capture by the liver. Estradiol, in contrast, greatly increased aminoisobutyrate up-

take by the proliferating uterus, and not by the liver. Growth hormone or epinephrine increased the amino acid concentration in a variety of tissues. These results imply that hormones modify amino acid metabolism by direct effects on the transport process. Direct effects on the destruction or anabolic utilization of the amino acid were ruled out by using an amino acid which underwent neither catabolism nor anabolism. In contrast, three hormones have been reported to modify transamination reactions: thyroxine, as mentioned elsewhere in this review; growth hormone, as reported by Zuchlewski & Gaebler (456); and adrenocortical hormones, as studied in lymphoid cell suspensions by Blecher & White (457)

Alanine transaminase has been reported to be inhibited by sulfonylurea derivatives, leading to the suggestion that these compounds lower blood sugar by slowing neoglucogenesis (458). Decreased alanine transaminase activity might not, however, decrease total neoglucogenesis.

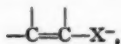
du Vigneaud and his associates (459, 460) have found that the inclusion of penicillamine in the diet of white rats causes the appearance of pyridoxine deficiency symptoms. The addition of this compound to liver transaminase systems, *in vitro*, caused an inhibition which was overcome by the further addition of pyridoxal phosphate.

Although most enzymes known to require pyridoxal phosphate as a coenzyme fail to exhibit a metal requirement for activity, it is possible that metal ions are tightly bound to the apoenzymes and therefore play the expected role in chelate formation. Beechey & Happold (461) have recently demonstrated that the interconversion of pyridoxal phosphate and pyridoxamine phosphate, catalyzed by an enzyme in *E. coli* extracts, requires  $Mg^{++}$  ions for maximal activity, and Happold & Turner (462) have found that the same metal stimulates sheep heart glutamic-oxalacetic carboxylase. Furthermore,  $Fe^{+++}$  ions are now known to be required for the decarboxylation of *p*-aminobenzoate in enzyme preparations from *E. coli* (463).

#### AMINO ACID AND AMINE OXIDASES

It is generally assumed that in the flavin-dependent dehydrogenation of an amino acid the imino acid is formed, which in turn reacts spontaneously with water to furnish the corresponding  $\alpha$ -keto acid and ammonia. Frieden & Velick (464) have incubated L-leucine in deuterium oxide with catalase and L-amino acid oxidase from rattlesnake venom and found that the resulting  $\alpha$ -keto acid was free of deuterium. This experiment rules out the possibility that an  $\alpha,\beta$ -dehydrogenated leucine is formed and therefore confirms the commonly accepted mechanism. Frisell & Hellerman (465) have observed, in accord with earlier work, that the sulfhydryl groups of D-amino acid oxidase are essential for enzymatic activity. Further study of the system has led them to conclude, however, that the sulfhydryl group is not needed for the actual binding of amino acids.

Kinetic analyses have shown that compounds with the anionic conjugate system,



are competitive inhibitors for amino acids in the D-amino acid oxidase system (466). Such structures are obviously similar in nature to the intermediate  $\alpha$ -imino acids. The inhibition of D-amino acid oxidase by *p*-amino salicylic acid has been studied in detail (467), and Klein (468) has proposed that the combination of FAD and the enzyme is an obligatory step prior to formation of the ternary complex containing FAD, protein, and inhibitor. According to Reddy (469), the inhibition of this enzyme by tumor extracts may be due to the action of a pyrophosphatase which splits FAD to FMN.

Radhakrishnan & Meister (470) have recently provided a clear demonstration of the reversibility of the amino acid oxidase reaction by experiments in which an  $\alpha$ -keto acid incubated anaerobically with D-amino acid oxidase, FAD, and a source of ammonia, furnished the corresponding D-amino acid. An oxidase in microorganisms which is specific for D-glutamic and D-aspartic acids has been highly purified by Mizushima & Sakaguchi (471). Otani & Meister (472) have demonstrated the oxidation of a series of  $\alpha$ -L-aminodicarboxylic acids and derivatives, such as  $\beta$ -aspartylalanine and  $\gamma$ -glutamylalanine, by opbio L-amino acid oxidase.

Zeller and his associates (473, 474, 475) have recently shown that monoamine oxidases and diamine oxidases are not as specific as their names would indicate, for both enzymes attack both mono- and diamines, although at different rates. Goryachenkova (476, 477) has observed that joint participation by FAD and pyridoxal phosphate is required for the action of diamine oxidase on histamine and other substrates, but the detailed mechanism of the reaction has not been elucidated. In accord with these findings, Davison (478) has reported that pig kidney diamine oxidase and histaminase are identical and that pyridoxal phosphate serves as the coenzyme, with the participation of FAD at some stage of the reaction not excluded. Tanaka (479) has reached similar conclusions in a study of the oxidative decomposition of amines by *P. aeruginosa*.

#### PYRIDINE NUCLEOTIDE-LINKED AMINO ACID DEHYDROGENASES

Snoke (480) has recently obtained crystalline glutamic dehydrogenase from chicken liver by a more simple isolation procedure than used by Olson & Anfinsen, or by Strecker, for the beef liver enzyme. The enzymes from the two sources have closely similar properties. Fincham (481) has obtained a modified glutamic acid dehydrogenase from a mutant derived secondarily from a strain of *N. crassa* lacking this enzyme. Unlike the dehydrogenase of the wild type organism, this modified enzyme exhibits significant activity only after it is warmed at 35 to 50° for a few minutes or preincubated with the substrates. Such cases of a qualitative change in a specific enzyme, as a result of mutation, are rare. Zelitch (482) has noted the occurrence in tobacco leaves of a DPN-specific glutamic dehydrogenase, as well as dehydrogenases which apparently act on other compounds, such as glutamine, and Jacobi (483) has observed the DPN-dependent deamination of both aspartic and glutamic acids by enzymes of *Ulva lactuca*. Levy & Vennesland (484) have extended earlier work in their laboratory, with deuterium as a

tracer, to show that liver glutamic dehydrogenase, like muscle glycerophosphate dehydrogenase, catalyzes the transfer of hydrogen with  $\alpha$ -stereospecificity for DPN.

In a study of possible glutamic dehydrogenase inhibitors, Caughey *et al.* (485) have observed that glutarate and  $\alpha$ -ketoglutarate are powerful competitive inhibitors and that thyroxine and related compounds inhibit both the forward and reverse reactions in an uncompetitive manner, but that D-amino acid oxidase inhibitors have no effect. Dunn *et al.* (486) have obtained preliminary evidence that *E. coli* contains a TPN-specific phenylalanine dehydrogenase. Kielley (487, 488) has made the interesting finding that the carcinogen, N-2-fluorenyldiacetamide, inhibits DPN-linked oxidations, notably glutamate oxidation, in rat liver mitochondria. The carcinogen apparently acts as a powerful competitor of DPN for glutamic dehydrogenase, but for some unknown reason does not exert this effect in the presence of the crystalline dehydrogenase. Emmelot (489) has noted the action of N-2-fluorenylacetamide in inhibiting glutamic dehydrogenase, and, to a lesser extent,  $\beta$ -hydroxybutyric dehydrogenase, in mouse liver mitochondria.

#### NITROGEN FIXATION AND NITRATE ASSIMILATION

*Nitrogen fixation.*—Although there is still no final answer to the question of whether hydroxylamine is an intermediate in nitrogen fixation, evidence continues to accumulate that ammonia is one of the primary products. Burris and his associates (490, 491) have found that the distribution of  $N^{15}$  in the nitrogenous products is strikingly similar when cells of *Azotobacter vinelandii*, which have been actively fixing nitrogen, are exposed to either  $N^{15}$ -ammonia or  $N_2^{15}$ . The brief lag period when ammonia is employed is believed to represent the time required for the ammonium ion to enter the cells and saturate the intracellular pool. In support of this view, cell-free preparations did not exhibit this lag period (492). Kinetic data obtained in these studies indicate that ammonia is the primary product of the fixation. In a study of the fate of isotopically labeled hydrazine in *Azotobacter*, Bach & Burris (493) have detected both  $N^{15}$ -ammonia and  $N_2^{15}$ . Sixty to 90 per cent of the hydrazine which had been utilized could be recovered as dihydropyridazinone-5-carboxylic acid and two other unidentified compounds, all of which apparently were formed by the chemical reaction of  $\alpha$ -ketoglutarate and hydrazine. The dihydropyridazinone carboxylic acid was found to occur naturally in soy bean nodules, and isotopic data suggest that it may serve as an intermediate in nitrogen fixation.

One of the chief obstacles in proving a role for the enzyme hydrogenase in the reduction of nitrogen has been the inability to show hydrogenase activity in the nodules of leguminous plants. Indirect spectrophotometric evidence has now been obtained, however, for the presence of this enzyme in sonic extracts of soybean nodules (494). Furthermore, Hoch, Little & Burris (495) have now provided a more direct demonstration of hydrogenase activity in this plant tissue.

Other investigations have supported the role of molybdate in nitrogen fixation (496, 497). The inhibition by tungstate of nitrogen fixation in *Azotobacter* was shown to be competitive with molybdate. Vanadium, which has been implicated in the past as replacing molybdenum in nitrogen fixation was ineffective in overcoming tungstate inhibition. This obviously suggests that vanadium cannot replace the molybdate requirement. Other workers have indicated inhibition of nitrogen fixation by azide (498) and by phosphite (499). In addition, several new nitrogen-fixing organisms have been reported (500, 501, 502).

The difficulty in consistently obtaining cell-free extracts which will fix nitrogen at a rate comparable to that of whole cells has been a major obstacle in studying the mechanism of this process. Recently, however, Nason and his associates (503) have obtained cell-free preparations of *Azotobacter* by sonic oscillation which catalyzed the fixation of  $N_2$ <sup>15</sup> at 25 to 100 per cent of the rate in intact cells. These preparations contained approximately 5 to 10 per cent unbroken cells, which may have acquired a considerably increased rate of nitrogen fixation. These workers have been able to show that dilution of *Azotobacter* cells results in as much as a twentyfold increase in nitrogen fixation, on a per cell basis.

**Nitrate assimilation.**—The reduction of nitrate to ammonia in many microorganisms and plants is now recognized to proceed stepwise by the successive gain of two electrons. The expected intermediates in this series of reactions are nitrite, possibly hyponitrite or nitramide, and hydroxylamine. Recent work has centered around the isolation and characterization of the enzymes responsible for these reactions.

Kinsky & McElroy (504) have found that the highly purified TPNH-specific nitrate reductase of *N. crassa* (505) is stimulated by phosphate. This enzyme is known to contain molybdenum, and it has been suggested that a phosphomolybdenum complex may be involved in the reaction. Nicholas & Scawin (506) have also observed the phosphate requirement for *Neurospora* nitrate reductase and have noted that phosphate can be completely replaced by arsenate, tellurate, or selenate. They postulate that the function of these anions is to bind molybdate to the apoenzyme. Preparations of this enzyme also contain a TPNH-cytochrome-*c* reductase which is believed to be intimately associated with the nitrate-reducing mechanism (504). Sadana & McElroy (507) have obtained spectrophotometric evidence that nitrate reductase, purified 260-fold, from *Achromobacter fischeri* contains a cytochrome which participates in nitrate reduction. In contrast to these observations with a mold and a bacterium, Silver (508) has reported that cytochromes do not play a direct role in nitrate reduction by the yeast *Hansenula anomala*.

Nitrite reductase has not been characterized as well as nitrate reductase. Nason and co-workers (509, 510, 511) have shown that both nitrite and hydroxylamine reductases of *Neurospora*, soybean, and *Azotobacter* are metallo-flavoproteins and that the enzymes from soybean and *Azotobacter* have a specific manganese requirement. Medina & Nicholas (512) have re-

cently reported that nitrite reductase of *Neurospora* is a metallo-flavoprotein containing either iron or copper. The latter information is based on the decrease in activity of preparations from iron- or copper-deficient cells. Such evidence obviously does not exclude the possibility that these metals play a role in the synthesis of the enzyme rather than participating as metal activators for enzyme activity. Spencer *et al.* (510) have observed that the corresponding enzyme system of *A. vinelandii* can utilize either DPNH or TPNH as the electron donor and that the flavin requirement for nitrite reductase is met specifically by FAD.

The most elusive intermediate in the over-all conversion of nitrate to ammonia is the compound between nitrite and hydroxylamine. If it is assumed that each step in nitrate reduction results in the gain of two electrons, the product expected here would be at the oxidation level of hyponitrite. McNall & Atkinson (513) have shown that *E. coli* B<sub>12</sub>, which utilizes nitrate or nitrite as the sole nitrogen source, can also utilize hyponitrite or hydroxylamine for growth. Furthermore, Medina & Nicholas (514) have demonstrated the presence of an enzyme in *Neurospora* extracts which will reduce hyponitrite to hydroxylamine by utilizing DPNH as the electron donor. This enzyme, like nitrite reductase, appears to be a metallo-flavoprotein, possibly containing iron or copper. Hyponitrite reduction was found to be inhibited by uncoupling agents such as dinitrophenol, and the authors suggest that a phosphorylation step is involved in the reduction. This does not necessarily implicate a phosphorylation step, however, since dinitrophenol usually stimulates electron transfer.

Roussos *et al.* (515) have found that the reported "ammonium dehydrogenase" activity (516) is an artifact, since the reduction of endogenous substrates was enhanced at the higher pH upon the addition of ammonium hydroxide. The conclusion that ammonium ion is not readily oxidized to hydroxylamine is in accord with the known equilibrium constant for hydroxylamine reduction (about  $10^{35}$ ) (515).



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# ENZYMOLGY OF NUCLEIC ACIDS, PURINES, AND PYRIMIDINES<sup>1,2</sup>

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Previous reviews have emphasized structural and metabolic studies in the field of nucleic acids, except that two years ago Carter (31) prepared an excellent account of the enzymic reactions in the synthesis of purine and pyrimidine nucleotides and of polynucleotides. The present review is limited strictly to the enzymology of nucleic acids, with emphasis on enzymic specificities, and to a discussion of recent progress in the enzymology of purines and pyrimidines.

## RIBONUCLEASES

### PANCREATIC RIBONUCLEASE

Pancreatic ribonuclease, first crystallized by Kunitz (112), continues to be the most intensively studied of all ribonucleases. As pointed out clearly some years ago by Schmidt *et al.* (180) and by Loring *et al.* (127) this enzyme catalyzes the cleavage only of certain strictly defined internucleotide bonds, namely those between the 3'-pyrimidine nucleoside phosphoryl groups and the 5'-hydroxyl groups of the adjacent purine or pyrimidine nucleoside residues. The specificity was nicely demonstrated by Brown & Todd (21) in experiments with simple esters of 2'- and 3'-monoribonucleotides which showed that only pyrimidine nucleoside 3'-alkyl phosphates were hydro-

<sup>1</sup> The survey of the literature pertaining to this review was completed in November, 1957.

<sup>2</sup> The following abbreviations are used for derivatives of inosine (I), adenosine (A), guanosine (G), uridine (U) and cytidine (C), respectively: The nucleoside-5' diphosphates are IDP, ADP, GDP, UDP and CDP. In representing polynucleotides, a phosphate group is denoted by "p"; when placed to the right of the nucleoside symbol, the phosphate is esterified at carbon 3' of the nucleoside moiety; when placed to the left of the nucleoside symbol, esterification is at carbon 5'. Thus, the two oligoribonucleotides at the bottom of Fig. 1 are abbreviated as pApApA and ApApUp. The oligodeoxyribonucleotide, TpTpT contains three thymidine residues linked by two 3':5' phosphodiester bridges, but no phosphomonoester groups. Polymers formed from nucleoside-5' diphosphates by the action of bacterial polynucleotide phosphorylase are designated poly A (made from ADP), poly U (made from UDP), poly AU (made from a mixture of ADP and UDP) etc. Other abbreviations used are: DNA for deoxyribonucleic acid; RNA for ribonucleic acid; TPN for triphosphopyridine nucleotide; and TPNH for triphosphopyridine nucleotide (reduced form).



lyzed. At least one alteration in the pyrimidine base is not allowable, for Magrath & Brown (134) observed that N-methyl uridine 3'-methyl phosphate was not attacked. On the other hand, incubation of RNA from tobacco mosaic virus which had incorporated 2-thiouracil-S<sup>35</sup> liberated 37 per cent of the radioactivity as thiouridylic acid (136). This implies that replacement of uracil by 2-thiouracil does not prevent the usual action of pancreatic ribonuclease. Further evidence that the enzyme is not limited in its specificity to uracil and cytosine derivatives is supplied by the independent discovery by Davis & Allen (40) and by Cohn (35) of an interesting fifth base in yeast RNA.<sup>2</sup> This new pyrimidine is a component of a nucleotide liberated by pancreatic ribonuclease. Finally, one should mention the work of Zamenhof *et al.* (201) showing that ribonuclease slowly hydrolyzes polyribose phosphate. Takemura (192) reported the hydrazinolysis of yeast RNA to form ribo-apyrimidinic acid. It will be of interest to see what the enzyme does to this material, which contains no pyrimidine bases.

Because of its sharp specificity, pancreatic ribonuclease acts on RNA to form 3'-UMP, 3'-CMP and a series of oligonucleotides consisting of purine nucleoside units and terminated by a pyrimidine containing residue. Many of these products were isolated in the pioneer investigations of Markham & Smith (138) and Volkin & Cohn (195). The polyribonucleotides synthesized with polynucleotide phosphorylase (see below) illustrate this specificity. Poly A and poly I are not hydrolyzed by ribonuclease, poly C and poly U give the 3'-mononucleotides in quantitative yield while poly AU gives 3'-UMP and a homologous series of oligonucleotides including ApUp, ApApUp, ApApApUp and ApApApApUp (89, 90). In certain RNA preparations, about 1 per cent of the nucleotide residues was present as terminal purine nucleoside 2':3' cyclic phosphoryl end-groups (139), isolated after exhaustive digestion with pancreatic ribonuclease. A surprising observation is that, in an experiment where the over-all replacement of guanine by azaguanine in RNA of *Bacillus cereus* was 22 per cent, the replacement in such cyclic purine nucleotides liberated by ribonuclease was 90 per cent (190). Also, the ratio of 8-azaguanine/guanine in the polynucleotides resulting from ribonuclease digestion increased with decreasing polynucleotide size. Structural differences among the RNA's from four strains of tobacco mosaic virus were observed, based upon variation in the amounts of pyrimidine mononucleotides formed by pancreatic ribonuclease (170).

Earlier work has shown that the action of ribonuclease proceeds in two phases, a transphosphorylation to form cyclic-ended oligonucleotides and pyrimidine nucleoside-2':3'-phosphates and subsequent hydrolysis of these compounds (138, 20). Certain inhibitors, such as heparin, sulphated pectins, and various polyphosphates were found to act selectively on the second phase thereby leading to an accumulation of cyclic derivatives (191, 194). The quantitative aspects of this biphasic action are shown in the hydrolysis of poly U and poly C (89). Thus, 0.03  $\mu$ g. of enzyme cleaved 25 per cent of the internucleotidic linkages in 1 mg. of poly U in an hour, at 37°, yielding a

series of cyclic-terminal oligonucleotides. Complete hydrolysis to 3'-UMP in one hour required fully 50  $\mu$ g. of enzyme.

Under certain conditions, synthetic capacities can be demonstrated for pancreatic ribonuclease (91, 92); for example, catalysis of the formation of cytidine-3'-methyl phosphate from cytidine-2':3'-phosphate and methanol. Barker *et al.* (6) have shown that only primary alcohols react in this enzymic conversion of nucleoside cyclic phosphates into nucleoside alkyl phosphates. This is consistent, as the authors point out, with the fact that in the synthesis of polynucleotides from cyclic-terminal mononucleotides, a 3':5'-diester linkage is established. They have also explored some interesting analogies between the action of ribonuclease and that of alkoxide ions, whose effect on RNA was previously investigated by Lipkin & Dixon (125).

Several years ago, Hirs, Moore & Stein (96) found that crystalline pancreatic ribonuclease is separable into a major "a" fraction and a smaller "b" fraction by chromatography on IRC-50 (XE-64) resin. Hakim has published a series of papers (73, 74) showing that the specificities usually attributed to the enzyme are actually a property of ribonuclease *a*. Ribonuclease *b* was reported to act quite differently, forming substantial amounts of 3'-GMP from RNA, as well as 3'-CMP and 3'-UMP. Some of his results are quite unexpected. For example, exhaustive digestion of RNA to ribonuclease *b* yielded guanine-containing, resistant oligonucleotides in amounts not very much different from those obtained with ribonuclease *a*, and yet ribonuclease *b* also liberated 14 per cent of the nucleotides of RNA as free guanylic acid. Ribonuclease *a* catalyzed a reversible exchange, or transesterification reaction, between cyclic-terminal pyrimidine nucleotides and nucleosides. By contrast, the *b* enzyme stimulated the irreversible synthesis, in nearly 100 per cent yield, of dinucleoside monophosphates from a mixture of nucleoside and cytidine-2':3'-phosphate, uridine-2':3'-phosphate or guanosine-2':3'-phosphate.

Hart (77) has analyzed certain experimental data of Reddi & Knight on the digestion of tobacco mosaic virus RNA by ribonuclease. The experimental results were found to be consistent with those which would be obtained with a randomly arranged ribonucleotide polymer of the same overall base composition as the viral RNA. The implication is that the sequential arrangement involves more than a very simple repeating unit. Morrill & Dickman (148) studied the properties of the *a* and *b* ribonucleases of mouse pancreas, both before and after stimulation by pancreozymin. Material was distributed among these two peaks quite differently, depending on the treatment. Roth (175) has continued the purification and characterization of the ribonuclease inhibitor from rat liver.

#### RIBONUCLEASES OTHER THAN PANCREATIC RIBONUCLEASE

Ribonuclease activity has been described in many plant and animal tissues. In some cases highly purified enzyme fractions have been obtained, but with a few exceptions (102, 140, 177) the specificity has not been de-

linedated. It is possible that fractions with new and interesting properties are available but these remain to be tested. For example, Roth (174) has purified an alkaline and an acid ribonuclease from rat liver mitochondria. The first of these resembles pancreatic ribonuclease in its stability to heat and  $H_2SO_4$  and may well have similar catalytic properties. Specificity studies on both fractions are now in progress.

Enzymic degradation of yeast ribonucleic acid by a heat stable fraction from *Aspergillus oryzae* yielded the four 3'-mononucleotides (111). In other work, a crystalline preparation with an acid pH optimum was obtained (176), but it was not homogeneous upon electrophoresis. More recently, Sato and Egami (177) separated and partially purified two ribonucleases from the same source. The first enzyme formed 3'-GMP rapidly from RNA and poly AGUC, but the other 3'-nucleotides were liberated exceedingly slowly, and poly A was scarcely attacked. This fraction was heat stable and showed a neutral pH optimum; a specific activity was achieved which approached that of pancreatic ribonuclease. The second fraction was also heat stable, with an acid pH optimum, and, in contrast to the first enzyme, formed much more 3'-AMP and pyrimidine mononucleotides than 3'-GMP in the early stage of digestion of RNA.

The ribonuclease from germinating rye grass has been purified 50-fold and separated from deoxyribonuclease and 3'-nucleotidase (184); it was observed to form mainly 5'-mononucleotides from RNA. A heat stable ribonuclease has been purified 700-fold from rat and guinea pig serum and separated from diphenyl phosphatase (167); it resembles the pancreatic enzyme in its pH-activity curve, thermostability and effect of heparin on activity. Maver & Greco were the first to point out the existence in animal tissues of ribonuclease and deoxyribonuclease activities differing from the pancreatic enzymes in pH optima,  $Mg^{++}$  inhibition and stability to heat. They have described the partial purification of fractions from spleen and liver, some of which have been separated into discrete peaks of activity by chromatographic techniques (141).

The specificity of a leaf ribonuclease (98) purified from pea and tobacco leaves has been investigated (140) and it was found to split all of the internucleotide linkages of RNA's to yield nucleoside-2':3'-phosphates. Of the latter, the pyrimidine derivatives were inert while the purine derivatives were slowly hydrolyzed to give nucleoside 3'-phosphates. The purine cyclic nucleotides also acted as donors in transesterification reactions involving nucleosides or nucleotides as acceptors. More recently, another purification of ribonuclease from tobacco leaves has been described (57) and its specificity investigated (171), with similar results. In addition, it was noted that among the early products of digestion there was a preponderance of guanosine-2':3'-phosphate and relatively little cytidine-2':3'-phosphate was formed.

## DEOXYRIBONUCLEASES

### PANCREATIC DEOXYRIBONUCLEASE

This enzyme, also called deoxyribonuclease I, is differentiated from the

deoxyribonucleases of class II because it is active in neutral solution and requires magnesium or certain other divalent cations. Its mode of action on DNA remains incompletely understood. The complex series of products include roughly one per cent of mononucleotides and 16 per cent of dinucleotides. A recent study (163) on isomeric dinucleotides derived from DNA agrees, in general, with earlier work (187); among those dinucleotides composed of one purine and one pyrimidine nucleotide the sequence pyrimidine nucleotide-purine nucleotide (p-Py-p-Pu) greatly predominated over the inverse sequence. Examination of the residue after dialysis against 0.02 M borate buffer, pH 7.3, showed a relative depletion in cytidylic acid (97). The mononucleotides are 5'-mononucleotides and the oligonucleotides are characterized by having a 5'-phosphomonoester end-group.

Polson (162) has devised an economical method for purification of the enzyme which combines the early steps in the Kunitz method (113) with the novel procedure of multimembrane electrodecentration. Klammerth (104) has demonstrated that deoxyribonucleoproteins of hen erythrocytes were more slowly degraded than protein-free DNA. Treatment of aqueous solutions of deoxyribonuclease with doses of x-irradiation sufficient to destroy the enzymic properties of this protein did not bring about an equally extensive change in the amino acid composition (156). The addition of DNA to an aqueous solution of deoxyribonuclease afforded potent protection against inactivation by x-irradiation (155). Proteins were also protective. Dounce *et al.* (42) have observed that pancreatic deoxyribonuclease, and also a mitochondrial deoxyribonuclease I, can penetrate cell nuclei during their isolation and thereby destroy their capacity to form gels in alkali or molar saline.

#### DEOXYRIBONUCLEASE II

This designation is applied to deoxyribonuclease activity with an acid pH optimum and no requirement for  $Mg^{++}$ . The inhibitor for deoxyribonuclease I is not active with deoxyribonuclease II; conversely, an inhibitor for deoxyribonuclease II has been found in human urine, and this does not inhibit deoxyribonuclease I (110). Shack (182) has found both types of deoxyribonucleases in a number of mouse tissues. The relative effectiveness of various salts in the activation of deoxyribonuclease II indicated that such activation resulted from binding of cations by DNA rather than from a non-specific increase of the ionic strength. Okada *et al.* (157) observed a rise in the deoxyribonuclease II activity in several organs of the rat after x-ray exposure; associated with this was a drop in the concentration of DNA. The enzyme activity has been purified from a number of sources.

(a) *Spleen*.—Maver and Greco first discovered deoxyribonuclease II in spleen and carried out a partial purification (141). Purification procedures involving the use of columns of Amberlite IRC-50 (XE-64) (109), and carboxymethyl-cellulose (183) have been devised. An important recent contribution by Koerner & Sinsheimer describes the preparation of highly purified enzyme (105) free of phosphatase and phosphodiesterase [measured with a bis-(*p*-nitrophenyl) phosphate salt]. When highly polymerized DNA

was digested with this enzyme the products were oligonucleotides with 3'-monoesterified phosphate end-groups (106). Independent discovery of this type of enzymatic attack on DNA was made by Cunningham *et al.* (39) (see section *b*). The splenic deoxyribonuclease showed no preference for specific purine or pyrimidine bases adjacent to the phosphodiester linkages hydrolyzed. Hydrolysis was rapid to a point where the average chain length of the polynucleotide products was 10; thereafter, hydrolysis continued at an ever-decreasing rate.

(*b*) *Micrococcus pyogenes*.—The enzyme was found in the culture medium and partially purified (39). Acting on DNA, it formed 3'-mononucleotides, and the dinucleotide ApCp has also been identified among the reaction products (163). Independent discovery of this new mode of attack on DNA was thus made in two laboratories. A third example of this type of hydrolysis of DNA is given in the next paragraph.

(*c*) *Thymus*.—Deoxyribonuclease from this tissue has been extensively purified by procedures which involve in part chromatography on columns, either of calcium phosphate (hydroxyapatite) (51) or of substituted cellulose (115). Laurila & Laskowski (115) have shown that the thymus enzyme forms 3'-mononucleotides in seven per cent yield from DNA. About nine per cent of the nucleotide units in the digestion mixture were accounted for as a trinucleotide and six dinucleotides, all with 3'-phosphomonoester end-groups. These results open the possibility that deoxyribonuclease II from all sources forms reaction products terminated in 3'-phosphate.

### PHOSPHODIESTERASES

In this review a phosphodiesterase is considered to be an enzyme fraction which hydrolyzes phosphodiester bonds, both in polyribonucleotides and polydeoxyribonucleotides. These fractions may turn out to be mixtures of enzymes. The two considered here have specificities which contrast in an interesting way.

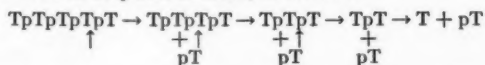
#### VENOM PHOSPHODIESTERASE

A number of fractionation procedures, most of them involving the use of chromatographic columns, have been worked out for the purification of venom phosphodiesterase and its separation from 5'-nucleotidase (18, 99, 188, 164). As first shown by Cohn & Volkin (36), such fractions hydrolyze RNA to give 5'-mononucleotides. Consistent with this, the enzyme hydrolyzes adenosine-5'-benzyl phosphate (5) but not the 3'-ester. It is a curious observation (41), although repeatedly confirmed, that nucleoside-2':3'-phosphates are slowly split to give nucleoside-3'-phosphates; perhaps this activity represents a minor, second enzyme. Venom phosphodiesterase is also active with the oligonucleotides formed from DNA by pancreatic deoxyribonuclease, which have 5'-phosphomonoester end-groups. These are converted quantitatively to deoxyribonucleoside-5'-phosphates (43, 100, 188). Similarly, ribopolynucleotides such as pApApA, formed from poly A by a fraction

from liver nuclei (88), as well as oligothymidine nucleotides such as pTpTpT, synthesized chemically (103) are rapidly hydrolyzed by the diesterase to give the corresponding ribo- or deoxyribonucleoside-5'-phosphates. Oligonucleotides bearing no phosphate end-group are also hydrolyzed by the venom fraction (106, 165, 195), although at a slower rate. Recent studies with a homologous series of oligothymidine nucleotides have shown compounds such as TpTpT are hydrolyzed at a twentieth of the rate of the corresponding 5'-phosphate-ended compounds (169).

Oligoribonucleotides which contain a 3'-phosphomonoester end-group are resistant to venom (106, 163, 195), although the presence of a 2':3'-cyclic phosphoryl end-group does not interfere with activity (138). This resistance is not absolute, for if the concentration of venom fraction is increased by several orders of magnitude, then hydrolysis occurs as indicated by the dotted lines of Fig. 1, forming nucleoside-3',5'-diphosphates among other products. These had been noted by Cohn & Volkin (36) but their origin was first made clear by Crestfield & Allen (38) who isolated pyrimidine-3',5'-diphosphates in amount equal to 60 per cent of the pyrimidine content of RNA, by treatment of a pancreatic digest with large amounts of venom. Similar relationships hold for the polydeoxyribonucleotides with 3'-phosphomonoester end-groups. A 1000-fold excess of venom over that needed to hydrolyze "5'-ended" deoxyribonucleotides was required to split ApCp to adenosine and pCp (163). Further, among the oligodeoxynucleotides with 3'-phosphomonoester end-groups, resistance to venom was less for compounds with a longer chain (106). The explanation offered is that a sufficiently long polymer chain can be broken at some link yielding a polynucleotide with no monoesterified phosphate and one with a 3'-end-group on one end of the chain and a 5'-end-group on the other (see Fig. 1). The former would be rapidly hydrolyzed; the latter might be further attacked from the end of the chain with the 5'-phosphate.

The higher rate of hydrolysis observed with dinucleotides bearing a 5'-phosphate end-group compared with dinucleoside monophosphates has led to the interesting suggestion by Laskowski (163) that venom phosphodiesterase may be comparable to carboxypeptidase, acting on the 5'-phosphomonoester end of a chain and removing 5'-mononucleotides by successive attack. Recently, observations have been made on a homologous series of thymidine nucleotides, TpT, TpTpT, TpTpTpT and TpTpTpTpT, all of which were hydrolyzed by venom phosphodiesterase at essentially the same rate (169). Careful analysis of the products of partial digestion revealed that hydrolysis occurred in stepwise fashion, as follows:



With thymidine oligonucleotides bearing a 5'-phosphate at one end and a 3'-acetyl group at the other, the first product to appear was the 3'-acetyl derivative of thymidine-5'-phosphate. These results confirm Laskowski's



hypothesis of a stepwise attack but, quite surprisingly, hydrolysis appears to begin, not at the 5'-phosphomono ester end of the chain, but at the opposite end. These results have been confirmed with oligoribonucleotides such as pApApApUpU . . . pU (see equation 2) which, in the initial stages of hydrolysis by venom phosphodiesterase, yielded only 5'-UMP (186).

#### SPLEEN PHOSPHODIESTERASE

It will be noted (Fig. 1) that this enzyme fraction, purified several hundredfold by Hilmoe (86), acts in a manner exactly opposite to that of the venom fraction. RNA is hydrolyzed to yield 3'-mononucleotides;

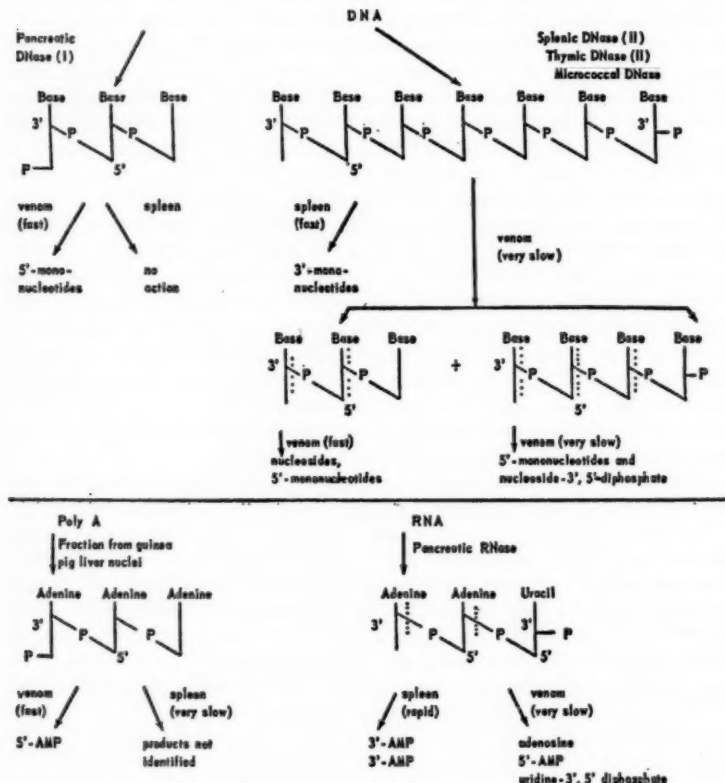


FIG. 1. The action of venom phosphodiesterase (18, 99, 188, 164) and of spleen phosphodiesterase (86) on polynucleotides containing a 5'- or a 3'-phosphomonoester end-group.



nucleoside-3'-alkyl phosphates are hydrolyzed, but not the 5'-esters. The "3'-ended" oligonucleotides remaining after exhaustive digestion of RNA or poly AU with pancreatic ribonuclease are rapidly split to 3'-mononucleotides (90), but "5'-ended" ribo-oligonucleotides are inert (88). Recently, Koerner & Sinsheimer found that deoxyribonucleotides with 3'-phosphomonoester end-groups are converted by this fraction, in good yield, to 3'-mononucleotides (106). The "5'-ended" deoxyribonucleotides, tested previously (86), were resistant. As with venom, there is evidence that if the level of enzyme is increased by several orders of magnitude, the "resistant" compounds show a slow attack.

A spleen fraction obtained by Maver & Greco (141) had similar properties, but did not hydrolyze polydeoxynucleotides. These specificities with respect to end-groups, as already noted, are less sharply defined or absent with large molecules. Thus, the biosynthetic polyribonucleotides (see below), which have 5'-phosphomonoester end-groups, are easily hydrolyzed by venom to give 5'-mononucleotides and by spleen to give 3'-mononucleotides (89, 90).

Neither the spleen nor the venom fractions show any specificity with respect to the purine or pyrimidine base. Also, N-1 methyl uridine-3'-methyl phosphate, which cannot be hydrolyzed by pancreatic ribonuclease (134), is acted upon by the spleen enzyme (94). A 2'-5'-phosphodiester linkage is required by spleen phosphodiesterase, so that A(2')p(5')U, a dinucleoside monophosphate with a 2'-5'-phosphodiester linkage between the adenosine and uridine moieties, is not hydrolyzed. This interesting compound was recently synthesized by Michelson, Szabo & Todd (143), who further reported that the same compound is easily hydrolyzed by venom phosphodiesterase to give 5'-UMP and adenosine.

## POLYNUCLEOTIDE SYNTHESIS

### SYNTHESIS OF RNA

The discovery of polynucleotide phosphorylase in *Azotobacter vinelandii*, by Grunberg-Manago & Ochoa (70), marked a notable advance in our understanding of the mechanism of RNA synthesis. The earlier work has been reviewed by Carter (31). This enzyme catalyzes the reversible synthesis of highly polymerized polyribonucleotides from 5'-nucleoside diphosphates, with release of orthophosphate:



It also catalyzes an exchange of  $\text{P}^{32}$ -labeled inorganic phosphate with the terminal phosphate group of nucleoside diphosphates. In the reverse direction, phosphorolysis of the polymers, and also of isolated RNA preparations takes place (7, 71, 126). Ochoa (153) has recently made a comparative study of the phosphorolysis of "natural" and "synthetic" polyribonucleotides. Polymers which contain a single base showed rapid phosphorolysis. The polymer containing four bases, and also isolated yeast and bacterial RNA's,

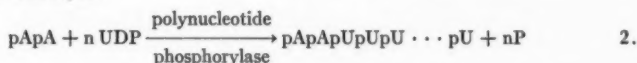
were slowly phosphorylated, it was suggested that the low susceptibility of some of these materials to phosphorolysis was due to the fact that they exist largely as multistranded chains.

A similar enzyme was purified from *Escherichia coli* by Littauer & Kornberg (126), who also observed the reversible synthesis of high molecular weight polymers, as well as the phosphorolysis of yeast RNA and several virus RNA's. An interesting finding was the several-fold stimulation of  $P^{32}$ -exchange reaction by a heat-stable fraction from *E. coli* [see discussion of this reaction by Kornberg (107)].

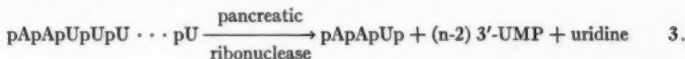
Polynucleotide phosphorylase has been fractionated from *Micrococcus leisodeikticus* by Beers (10) and by Olmsted (158). A method of isolation of DNA from this organism has been devised which depends on selective destruction of RNA in the crude extract by the polynucleotide phosphorylase which is contained in it (7). Beers has emphasized the complex interrelationships between ADP and  $Mg^{++}$  concentrations, pH and rate of polymer formation (8). He has also noted the need for rather high concentrations of salt (9), a requirement which has not been evident with highly purified azotobacter fractions. Particularly interesting is the observation that, while the initial rate of polymerization was proportional to ADP concentration up to the level of substrate saturation, the rate of polymerization was linear with time until equilibrium was reached, in spite of the falling concentration of substrate. Olmsted has reported evidence for several polynucleotide phosphorylases, active with different nucleoside diphosphates (158). With the most highly purified azotobacter fraction, no dissociation of activities was encountered (154). Singer (185) has made a careful study of the phosphorolysis of different kinds of oligonucleotides by the phosphorylases of *E. coli* and *A. vinelandii*, including in each series the di-, tri- and tetranucleotide. Compounds with a 5'-phosphomonoester end-group or with no end-group were readily phosphorylated, but those with a 3'-phosphomonoester end-group or a 2':3'-cyclic phosphoryl end-group were not attacked. It was noted that phosphorolysis proceeded only to the stage where the polynucleotide chain was reduced in length to 2 nucleoside residues. The dinucleotides and dinucleoside monophosphates were "limit polynucleotides," resistant to further breakdown, but they could serve as primers (see below).

A significant advance was made by the discovery of Mii & Ochoa (144) that with highly purified enzyme from *A. vinelandii*, polymerization of ADP showed an autocatalytic time curve, with a long initial lag period. This lag period was eliminated by poly A but not by poly U. Conversely, in the polymerization of UDP the lag period was eliminated by poly U, but not by poly A. Results with IDP also indicated that primer and nucleoside diphosphate should have a common base. Poly C stimulated the polymerization of CDP, UDP, ADP, IDP and the synthesis of poly AGUC (synthetic RNA). On the other hand, the synthesis of poly C was stimulated only by poly C and inhibited by all other polyribonucleotides including RNA. RNA primed the synthesis of poly AGUC as well as that of poly A and poly U. Although

the mechanism of the priming effect of polynucleotides is still unknown, the pattern of specificity observed is suggestive of a directing influence on the nature of the polynucleotide synthesized. Some of these results were confirmed by Singer *et al.* (185a) who found, in addition, that certain oligonucleotides also served to eliminate the lag period (with no specificity for the base). Compounds like pApA and pApApA were shown to be primers for the synthesis of both poly A and poly U in the strict sense of the word, because they were in fact incorporated into the polymer, serving as a nucleus for the chain. For example:



Evidence for reaction 2 was obtained by digestion of the isolated polymer with ribonuclease, followed by recovery and identification of the adenine containing primer with one uridine residue attached.



The occurrence of polynucleotide phosphorylase in extracts of a number of bacteria has been established (22). Small but definite activity was found in spinach leaf extracts (22). An enzyme has been partially purified from yeast which synthesizes phosphorylated polynucleotides starting with nucleoside diphosphates (69). The location of the extra phosphate is under investigation. The yeast fraction shows certain peculiarities (72); for example, the  $\text{P}^{32}$ -exchange is inhibited by  $\text{Mg}^{++}$  whereas the release of inorganic phosphate and the formation of polynucleotide-material requires  $\text{Mg}^{++}$ . Net phosphorolysis of bacterial poly A by a fraction of guinea pig liver has been reported (95) but as yet net synthesis of polynucleotide has not been demonstrated.

#### PROPERTIES OF THE SYNTHETIC POLYRIBONUCLEOTIDES

These polymers are nondialyzable, often viscous materials, of high molecular weight. It is possible to control their size by variation in the ratio of enzyme to substrate (52), by adding oligonucleotide primer, by variation in time of incubation, and by other means. Warner (197, 198) discovered that poly A and poly U interact in dilute neutral salt solutions to form a new species with higher sedimentation coefficient, and altered electrophoretic mobility and ultraviolet spectrum. X-ray diffraction patterns of fibers drawn from such mixtures have been interpreted in terms of a two-stranded helical structure, similar to the DNA molecule (173). More recently, a three-stranded structure has been discovered, formed by interaction of the complex of poly A and poly U with a second equivalent of poly U (46). Small cations are required for these reactions. It has been reported that the interaction of poly A and poly U does not occur in the region below pH 5 to pH 6 or in the region above pH 10 (12).

Several groups have observed an interesting transition when solutions of poly A are titrated with acid. Beers & Steiner (11) observed that a group is titrated in the region of pH 6, and the change is accompanied by alteration in the ultraviolet spectrum and changes in light scattering. Formaldehyde or calcium ions greatly reduce the binding of hydrogen ion commencing at approximately pH 6. The authors feel that the primary amino group of the adenine moiety is free in alkaline solutions and is in salt linkage with the phosphate group of a neighboring nucleotide at pH values less than 6. Fresco & Doty (52) have obtained evidence for the nature of the configurational change which this transition indicates. The molecular weight dependence of sedimentation coefficient and intrinsic viscosity were typical of randomly coiled single chains on the alkaline side of the transition points, but on the acid side the samples showed evidence of the formation of stable aggregates. Further, this form showed no marked increase in specific viscosity upon removal of salt, and its behavior in flow birefringence indicated a relatively rigid structure. These authors also noted that the spectral transition was sharp and complete. They have postulated, for the acid form, a structure in which poly A molecules are associated through hydrogen-bonded base-pairing in a double-stranded helix, each strand of which has gaps where one poly-A molecule ends and another begins. This model is consistent with the x-ray diffraction patterns obtained by Rich, Watson & Crick on fibers of poly-A (172, 199); and Fresco & Crick (53) have obtained x-ray diffraction patterns of poly A, as a function of pH, which has led them to identify the acid form of the polymer with the crystalline fiber diagram. Hall, Fresco & Doty (75) have also obtained electron microscope photographs of the random coil and helical (acid) form of poly A. By contrast, it was found that poly U exists as randomly coiled single chains over the entire pH range (81).

Pinchot (161) reported that a heat-stable factor required for oxidative phosphorylation in extracts of *Alkaligenes faecalis* could be replaced by various synthetic polyribonucleotides. Hart & Smith (78) found that certain natural RNA's and synthetic polyribonucleotides, including poly A, poly U, poly C and poly AGUC would interact with protein from tobacco mosaic virus to form virus-like particles which were non-infectious. An influence of poly A and other polynucleotides on the ATP level of isolated thymus nuclei has been reported (2). Like natural RNA, synthetic RNA (but not poly A, poly AU or poly AC) promoted the formation of streptolysin S by cells of *Streptococcus pyogenes* (193).

#### INCORPORATION OF ISOTOPES INTO RNA FRACTIONS WITH CELL-FREE SYSTEMS FROM ANIMAL TISSUES

Herbert, Potter & Hecht (93) have studied the incorporation of radioactivity from orotic acid 6-C<sup>14</sup> into RNA in cell-free systems from rat liver. Radioactivity could be recovered as the 2',3' uridine monophosphates following alkaline hydrolysis. Isotope dilution experiments suggested that 5'-uridine phosphate (or a derivative) was an intermediate but not the 2'- or

3'-mononucleotide. Studies with recombined cell fractions showed that a supernatant fraction was essential, presumably to convert orotic acid to uracil nucleotides. Stimulation of supernate by microsomes showed that microsomes could catalyze rate-limiting steps in the further incorporation into RNA. When whole homogenates were employed, the incorporation of radioactivity into both nuclear and cytoplasmic ribonucleic acids occurred and it was more rapid and reached a plateau sooner in the nuclear fraction than in the cytoplasmic fraction. Schneider & Potter (181) found that ATP caused a temporary inhibition of this incorporation process. Two groups of workers (3, 126a, 126b) have found that RNA of thymus nuclei was separable into two distinct fractions. These showed great differences in metabolic activity when the incorporation of orotic acid-6-C<sup>14</sup>, adenosine-8-C<sup>14</sup>-P<sup>32</sup> and adenine-C<sup>14</sup> were studied *in vitro*.

Canellakis (29) has studied the incorporation of UMP-4-C<sup>14</sup> into an RNA fraction, using dialyzed acetone powder extracts of the particle-free cytoplasmic fraction of rat liver. The incubation mixture was treated with perchloric acid, the precipitate extracted with 10 per cent NaCl and the RNA precipitated with alcohol. The radioactivity was then isolated in the form of uridine-3'-phosphate after digestion with ribonuclease, and as uridine-3'-(and -2') phosphate after alkaline hydrolysis. More recently, four groups of workers found that ATP is incorporated in a very special manner into RNA or RNA-like material. With the fraction just mentioned, Canellakis (30) observed that an internucleotide linkage was formed and AMP was attached to a terminal monoesterified cytidylic acid of RNA. The system would not attach AMP to other nucleotides in RNA. Earlier work by Heidelberger *et al.* (85) had already shown that when AMP<sup>32</sup> was incubated with rat liver homogenate and the RNA hydrolyzed with alkali, the P<sup>32</sup> was bound with cytidine-2'- and 3'-phosphate. Zamecnik *et al.* (200), working with a pH 5.2 precipitate of the 105,000 g supernatant of rat liver homogenate also noted that over 90 per cent of the AMP from ATP-8-C<sup>14</sup> was located in a terminal position, so that it appeared as adenosine following alkaline hydrolysis. Similar observations were made by Paterson & LePage (159), using a cytoplasmic fraction obtained by high-speed centrifugation of homogenates of Flexner-Jobling carcinoma. Edmonds & Abrams (44a), working with soluble fractions of Ehrlich ascites carcinoma, have evidence suggesting that separate enzymatic mechanisms may be responsible for two types of incorporation of ATP-8-C<sup>14</sup>, one leading to liberation of C<sup>14</sup> as adenosine, and the other to liberation as a mixture of 2'-AMP and 3'-AMP upon alkaline hydrolysis.

#### SYNTHESIS OF DNA

The brilliant studies of Kornberg *et al.* (14, 107) have clarified the enzymatic biosynthesis of DNA. An enzyme has been purified 1000-fold from extracts of *E. coli*, which catalyzes the incorporation of deoxyribonucleoside-triphosphates, labeled with P<sup>32</sup> in the 5'-phosphoryl group, into the acid insoluble fraction. The system requires all four triphosphates, a highly poly-

merized DNA and  $Mg^{++}$ . Thymidine diphosphate could not replace thymidine triphosphate. Actually, omission of any one of the four triphosphates did give a measurable rate, but it was only 1 per cent of that of the complete system. Net synthesis has not exceeded 50 per cent of the amount of DNA originally added, but this yield is expected to improve with the removal of interfering activities from the enzyme preparation. It remains to be determined whether the DNA chains added as "primer" have radioactive nucleotide units added onto them, or serve as templates for new chains. Inorganic pyrophosphate is formed in the reaction, equivalent in amount to the nucleoside triphosphates incorporated. The system is relatively irreversible, although with  $10^{-3}$  *M* labeled inorganic pyrophosphate, an exchange with triphosphate was shown, and this was dependent on the presence of DNA, enzyme and  $Mg^{++}$ . Very impressive is the fact that all four triphosphates are required for effective incorporation, and substrate affinities are so great that concentrations of only  $10^{-5}$  *M* are employed.

Kornberg (107) has discussed two alternative mechanisms of polymerization. In mechanism (A), the polynucleotide chain (or acceptor group) ends as a pyrophosphate and successive nucleoside triphosphates would be added to this end, with displacement of inorganic pyrophosphate from the acceptor. In what he considers to be the more plausible mechanism (B), the nucleoside diphosphate adds to the carbohydrate end (terminal nucleoside residue) of the acceptor polynucleotide with displacement of inorganic pyrophosphate from the donor. It is of interest that in the reaction shown in equation 2 (see above) catalyzed by polynucleotide phosphorylase, it was found that every chain was built upon the pApA primer. In this case, involving polynucleotide synthesis, the chains were able to grow only by mechanism B.

With respect to subcellular fractions from animal tissues, incorporation of thymidine- $C^{14}$  into DNA of rabbit thymus nuclei has been demonstrated (56) and, more recently Bollum & Potter (17) have succeeded in demonstrating the incorporation of tritium labeled thymidine into DNA homogenates prepared from 24-hour regenerating livers of rats. Low incorporation was obtained at other time intervals, which argues against a simple exchange mechanism. The activity has now been found in the supernatant fraction, and addition of DNA was required to bring about incorporation of tritium-labeled thymidine into the reisolated DNA (16).

## PURINE METABOLISM

### INOSINIC ACID BIOSYNTHESIS

Previously published reviews by Carter (31), Buchanan *et al.* (23) and Greenberg & Jaenicke (65) have summarized the pathway of inosinic acid biosynthesis. The purification of several of the enzymes and the isolation of several of the intermediates of this pathway have been reported during the past year.

The enzyme catalyzing the conversion of ( $\alpha$ -N-formyl)-glycinamide ribotide (79) to ( $\alpha$ -N-formyl)-glycinamide ribotide (120) has been purified



from pigeon liver homogenates about 45-fold [Melnick & Buchanan (142)]. The product of the reaction was determined after conversion to 5-aminoimidazole ribotide with a partially purified protein obtained from the same homogenate. The imidazole derivative formed is readily determined in the Bratton-Marshall reaction for arylamines. Requirements for ( $\alpha$ -N-formyl)-glycinamide ribotide, ATP, glutamine,  $Mg^{++}$ , and  $K^+$  were demonstrated. The specificity of the nucleotide, amide and cation requirements was not reported. Glutamic acid was identified qualitatively as a reaction product. The enzyme preparation is free of adenylate kinase. Thus, the fact that ADP and no AMP is formed indicates that the products of the reaction are ADP and orthophosphate rather than AMP and pyrophosphate.

The ribotide formed in this reaction has been purified by ion exchange chromatography and isolated as a barium salt in an over-all yield of 11 per cent based on the amount of ( $\alpha$ -N-formyl)-glycinamide ribotide used [Levenberg & Buchanan (120)]. Hydrolysis in acid or alkali liberates glycine, formate, pentose, phosphate, and ammonia in the ratio of 1:1:1:1:2 based on glycine; the total N found after combustion was three moles per mole of glycine. Titration indicates the presence of two dissociable groups with  $pK'$  values of 6.0 and 9.2, which are thought to correspond to the dissociation of the secondary phosphate and the amidine group respectively. The material does not react in either the Pauly diazo reaction or the Bratton-Marshall test and shows only weak absorption below 240  $m\mu$ . All these observations are consistent with the absence of the imidazole ring and with the structure of ( $\alpha$ -N-formyl)-glycinamidine ribotide.

When the isolated product is incubated with a partially purified preparation from pigeon liver and ATP or ADP, it is converted to an arylamine, presumably 5-aminoimidazole ribotide [Levenberg & Buchanan (121)]. The same product was formed from ( $\alpha$ -N-formyl)-glycinamide ribotide, glutamine and ATP and was purified by chromatography on Dowex-1-acetate. The barium salt was isolated and purified in an over-all yield of 25 per cent. The material was estimated to be from 35 to 60 per cent pure. The instability of the ribotide limited attempts to obtain the compound in pure form. The isolated product contained acetate and inorganic salts. The ratio of glycine, formate, pentose, phosphate, and ammonia liberated by hydrolysis was 1:1:1:1:2; and the total N found after combustion was three moles per mole of glycine. However, this product, in distinction to the isolated ( $\alpha$ -N-formyl)-glycinamidine ribotide, reacts in the Bratton-Marshall test for diazotizable aromatic amines. The chromophore formed has an absorption maximum at 500  $m\mu$  by which it can be distinguished from the product formed from 5-amino-4-imidazolecarboxamide ribotide. 5-Aminoimidazole ribotide shows no absorption maximum in the range 210 to 300  $m\mu$ . The aglycone of this ribotide, 4(5)-aminoimidazole, previously described as an intermediate in the fermentation of purines by *Clostridium cylindrosporium* and *Clostridium acidi-urici*, was found to have a molecular extinction coefficient of 3800 at its absorption maximum at 238  $m\mu$  in 0.1 N HCl or at pH



5.0 [Rabinowitz (168)]. The aglycone was found to be particularly susceptible to decomposition in the presence of oxygen.

5-Aminoimidazole ribotide is converted to 5-amino-4-imidazole carboxamide ribotide upon incubation with ATP, aspartate, and bicarbonate by two partially purified enzyme fractions [Lukens & Buchanan (129)]. When 5-aminoimidazole ribotide is incubated with the first of these enzyme fractions in the presence of bicarbonate alone, another diazotizable amine is formed, which in contrast to the starting imidazole derivative, shows an absorption maximum at  $249\text{ m}\mu$  at pH 8.5. The chromophore formed by this product in the Bratton-Marshall reaction has a maximum absorption at  $519\text{ m}\mu$ . This substance is presumed to be 5-amino-4-imidazolecarboxylic acid ribotide. In previous work, both the aglycones involved in this reaction were encountered as products of the fermentation of xanthine [Rabinowitz (168)]. 5-Amino-4-imidazolecarboxylic acid was found to have an absorption maximum at  $260\text{ m}\mu$ , and the absorption maxima of the chromophores formed from synthetic samples of 4-aminoimidazole, 5-amino-4-imidazolecarboxylic acid, and 5-amino-4-imidazolecarboxamide in the Bratton-Marshall test carried out in 5 per cent perchloric acid were at 514, 502, and  $518\text{ m}\mu$ , respectively (168). The absorption maxima of the Bratton-Marshall reaction products of the ribotides of these compounds in 15 per cent trichloroacetic acid occur at 500 (121), 519 (129) and  $555\text{ m}\mu$  (121), respectively. The differences observed are probably attributable to the different acid strengths used. The ribotide of 5-amino-4-imidazolecarboxylic acid as well as the aglycone are easily decarboxylated in the presence of acid (121, 168).

The first enzyme fraction, which catalyzes the formation of 5-amino-4-imidazolecarboxylic acid ribotide from 5-aminoimidazole ribotide and bicarbonate, also catalyzes the formation of 5-amino-4-imidazole-N-succinocarboxamide ribotide (128, 129) from 5-aminoimidazole ribotide, ATP, aspartate, and bicarbonate. ADP and orthophosphate were identified as the other reaction products. Other nucleoside triphosphates are less active than ATP. The conversion of 5-amino-4-imidazole carboxylic acid ribotide to 5-amino-4-imidazole-N-succinocarboxamide ribotide was not demonstrated directly. However, 5-amino-4-imidazole-carboxylic acid ribotide is converted to 5-amino-4-imidazolecarboxamide ribotide by a mixture of the two enzyme fractions incubated with ATP and aspartate in the absence of bicarbonate [Lukens & Buchanan (129)].

The second enzyme fraction, which catalyzes the cleavage of 5-amino-4-imidazole-N-succinocarboxamide ribotide, has been purified from pigeon or chicken liver, as well as from various bacterial sources [Miller, *et al.* (145)]. The products formed by the purified enzyme are 5-amino-4-imidazolecarboxamide ribotide and fumaric acid. The fact that the purified enzyme also splits adenylosuccinic acid (32), and the ratio of splitting of these two substrates remains constant during purification from yeast, suggests that the same enzyme acts on both substrates. The  $K_m$  of yeast adenylosuccinase for adenylosuccinate is  $1.2 \times 10^{-5}\text{ M}$  [Carter & Cohen (32)], while the  $K_m$  for

5-amino-4-imidazole-N-succinocarboxamide ribotide is  $1.9 \times 10^{-4} M$  (145).

The ribotide of 5-amino-4-imidazolecarboxamide has also been obtained by two other enzymatic routes. A nucleotide pyrophosphorylase which catalyzes the reaction between the imidazole and 5-phosphoribosyl pyrophosphate has been purified from acetone powders of beef liver [Flaks *et al.* (49)]. Adenine as well as 4-amino-5-imidazole carboxamide acts as a substrate. The  $K_m$  for adenine is too low to be determined, while that for the imidazole derivative is  $3.1 \times 10^{-4} M$  (49). Adenosine-5'-phosphate pyrophosphorylase, previously purified from yeast, is also active with hypoxanthine and guanine; however, the activity of 4-amino-5-imidazolecarboxamide or its ribotide was not determined (108). The nucleotide has also been prepared by enzymatic phosphorylation of the nucleoside obtained from sulfonamide inhibited *E. coli* (66), using a dialyzed yeast autolysate [Greenberg (64)].

The conversion of 5-amino-4-imidazolecarboxamide ribotide to inosinic acid has been proposed for the final stages of purine biosynthesis (50, 63). The reaction is most easily studied in the reverse direction by determination of the nonacetyltable arylamine formed from inosinic acid. Upon purification of the enzyme from pigeon or chicken liver, stimulation of the reaction by glycine, a reduced folic acid derivative, malic acid and TPN were demonstrated (50). Serine and 5-amino-4-imidazolecarboxamide were identified as reaction products. It has not yet been possible to demonstrate the formation of the presumed intermediate of this reaction, 5-formamido-4-imidazolecarboxamide ribotide in studying the reaction from either direction (23). It will probably first be necessary to free the enzyme preparations of inosinase, which rapidly effects the cyclization of synthetic 5-formamido-4-imidazolecarboxamide ribotide to inosinic acid in the absence of ATP [Buchanan *et al.* (23)].

#### PURINE ANALOGUES AND INHIBITORS

L-Azaserine (O-diazoacetyl-L-serine) inhibits purine biosynthesis by pigeon liver extracts (80), and has been shown to exert a strong inhibitory effect on the conversion of ( $\alpha$ -N-formyl)-glycinamide ribotide to ( $\alpha$ -N-formyl)-glycinamidine ribotide [Levenberg *et al.* (119, 122)], as well as a smaller inhibitory effect on the enzymatic conversion of ( $\alpha$ -N-formyl)-glycinamidine ribotide to 5-aminoimidazole ribotide and on the synthesis of 5-phosphoribosylamine from glutamine and 5-phosphoribosylpyrophosphate by partially purified pigeon liver fractions (122). 6-Diazo-5-oxo-L-norleucine is an even more effective inhibitor than azaserine. The inhibition caused by both compounds can be almost completely reversed by glutamine in a competitive manner. However, noncompetitive inhibition is observed when either inhibitor is preincubated with the enzyme in the absence of glutamine (122).

The specific mechanism of action of 6-mercaptopurine is still not known. The ribotide of 6-mercaptopurine was prepared from the base and 5-phosphoribosylpyrophosphate with an enzyme purified from beef liver acetone powder [Lukens & Herrington (130)]. Neither the free base nor the ribotide

inhibited the synthesis of inosinic acid from glycine by a soluble pigeon liver system (130).

The 8-azapurines are incorporated into the RNA of susceptible bacterial species (135, 137, 190). A very good correlation between the inhibitory action of the 8-azapurines and their incorporation into the bacterial RNA as 8-azaguanine has been demonstrated [Smith & Matthews (190)]. This suggests that the inhibition caused by these analogues is due to the inability of the RNA containing 8-azaguanine to function normally, rather than to the inhibition of specific enzymes involved in purine metabolism by the analogue itself.

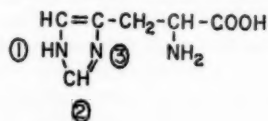
#### GUANYLIC ACID BIOSYNTHESIS

Inosine-5'-phosphate dehydrogenase has been purified from extracts of *Aerobacter aerogenes* by Magasanik *et al.* (132). The presence of a similar enzyme had previously been demonstrated by Abrams & Bentley in bone marrow extracts (1) and by Lagerkvist in pigeon liver (114). The product of the purified bacterial enzyme was isolated and characterized as xanthosine-5'-phosphate. DPN<sup>+</sup> is required as the oxidant, and TPN<sup>+</sup> is inactive. Stimulation of the enzyme by thiols was sometimes observed, and requirements for K<sup>+</sup> or NH<sub>4</sub><sup>+</sup> were demonstrable. No evidence for reversibility of the reaction could be obtained.

Xanthosine-5'-phosphate aminase, an activity previously demonstrated in animal sources (1, 114), has been purified about 300-fold from *A. aerogenes* by Moyed & Magasanik (149). Xanthosine-5'-phosphate, ATP, Mg<sup>++</sup>, and an ammonium salt are required for the reaction. The products formed by the bacterial enzyme have been identified as guanosine-5'-phosphate, adenosine-5'-phosphate, and inorganic pyrophosphate. Reversal of the reaction could not be demonstrated. Glutamic acid or glutamine can also serve as the N donor with crude bacterial extracts, but the activity with glutamine is lost upon purification and the reaction is specific for ammonia. The *K<sub>m</sub>* for NH<sub>4</sub><sup>+</sup> at pH 7.15 is 0.1 *M*. However, evidence is presented suggesting that free ammonia is the actual amino donor, and the calculated *K* for NH<sub>3</sub> at pH 7.15 is  $0.96 \times 10^{-3}$  *M*. The reaction is not reversible. The amino donor for the xanthosine-5'-phosphate aminase of rabbit bone marrow has been shown to be the amide group of glutamine and ammonia is inactive in the system [Bentley & Abrams (13)].

#### PURINES IN HISTIDINE BIOSYNTHESIS

Previous nutritional studies have indicated a role of purines in histidine biosynthesis [Broquist & Snell (19)]. Using isotopes, it was demonstrated



that C-2 of guanine is directly incorporated into the C-2 position of histidine by growing cultures of *Lactobacillus casei* (146) and a mutant of *E. coli* (133). This transformation was shown by Mitoma & Snell (146) to occur in the absence of added folic acid, and suggested that this one carbon transfer was not mediated by a folic acid derivative. Guanine was also found to contribute an N atom to histidine [Magasanik (131)]. Since there are three N atoms bonded to C-2 of guanine, it was of particular interest when it was shown by Neidle & Waelsch (152) that the amino group of guanine is not incorporated into histidine, but that either the N-1 or N-3 of guanine together with the C-2 of guanine<sup>3</sup> is converted to N-1 and C-2 of histidine; while N-3 of histidine is derived from the amide N of glutamine (151).

Earlier work by Ames & Mitchell (4) with *Neurospora crassa* had established the occurrence of D-erythro-imidazole-glycerol phosphate as one of the intermediates in the biosynthesis of histidine. The configuration of this compound is the same as that of D-ribose, and suggested that the five carbon chain of histidine is derived from ribose or a derivative such as ribose-5-phosphate.

These observations are nicely related by the scheme for histidine biosynthesis suggested by Magasanik & Moyed (149a) based on their findings using extracts of a histidineless mutant of *Salmonella typhimurium* (150). Imidazole glycerol phosphate and 5-amino-4-imidazolecarboxamide ribotide are formed upon incubation of the extracts with adenosine-5'-phosphate, ribose-5-phosphate, an ATP generating system, and glutamine. In the absence of glutamine, a compound accumulates which yields 5-amino-4-imidazolecarboxamide ribotide upon mild acid hydrolysis. In experiments with labeled substrates, it was found that ribose-5-phosphate-1-C<sup>14</sup> is incorporated into imidazoleglycerol phosphate without dilution; AMP-8-C<sup>14</sup> is converted to 5-amino-4-imidazolecarboxamide ribotide without dilution; AMP-2-C<sup>14</sup> is converted to imidazoleglycerol phosphate of the same specific activity, while the 5-amino-4-imidazolecarboxamide ribotide formed in this experiment does not contain any C<sup>14</sup> (149a).

A scheme summarizing these observations and the hypothetical structure of the intermediate is shown in Figure 2. The adenylic acid used as the C-N donor could be regenerated from the 5-amino-4-imidazolecarboxamide ribotide by known enzymatic reactions involved in adenylic acid biosynthesis. Thus adenylic acid would be required in catalytic amounts in this biosynthesis from ribose-5-phosphate, ATP, glutamine, aspartate, and an active one carbon donor. The N-1, C-2 and N-3 of histidine could be derived from the amino group of aspartic acid, the active one carbon compound, and the amide N of glutamine respectively. In the absence of folic acid, aspartic acid or the enzymes required for regenerating adenylic acid from 5-amino-4-imidazolecarboxamide ribotide, purines would be required in substrate

<sup>3</sup> The numbering of imidazole ring of histidine in the publications of Waelsch and co-workers (151, 152) is the reverse of that used in this review and in the publications of Magasanik (131).

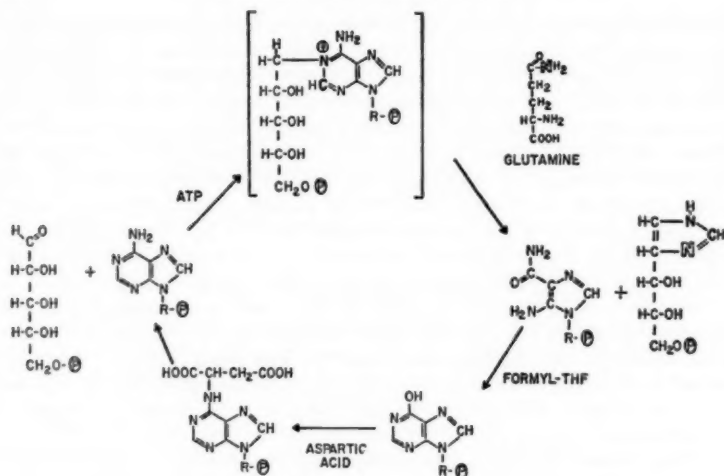


FIG. 2. Biosynthesis of imidazole glycerol phosphate: R = ribose,  $\text{P}$  = phosphate, formyl-THF = formyl-tetrahydrofolic acid.

amounts for histidine biosynthesis. The exact nature of the proposed intermediate formed from adenylic acid and ribose-5-phosphate (or possibly 5-phosphoribosyl pyrophosphate) remains to be established, as well as the nature of the enzymatic reactions involved in these transformations.

#### PURINE DEAMINASES

Adenine deaminase has been purified 35-fold from extracts of *Azotobacter vinelandii* (87). The purified enzyme preparation shows no activity with adenosine, guanine, or hypoxanthine, and can be used for a convenient spectrophotometric assay of adenine.

5'-Adenylic acid deaminase has been obtained in crystalline form from rabbit skeletal muscle by Lee (116, 117, 118), and shown to be a homogeneous protein. The crystalline preparation is free of adenine, adenosine, adenosine diphosphate, cytosine, and guanosine deaminase activities, as well as myokinase and adenosine triphosphate-creatine transphosphorylase.

#### PYRIMIDINE METABOLISM

##### URACIL

The enzymatic reactions involved in the conversion of aspartic acid to carbamylaspartic acid, dihydroorotic acid, orotic acid, orotidylic acid, and uridylic acid have been reviewed previously [Carter (31)], and this pathway is generally assumed to be responsible for the net synthesis of the pyrimidines. Uracil and thymine have been shown to be degraded through their

corresponding dihydropyrimidines, and carbamylamino acids to yield  $\beta$ -alanine and  $\beta$ -aminoisobutyric acid when fed to animals or when incubated with tissue slices (47, 48, 58). Several of the enzymes involved in the metabolism of uracil and thymine have now been purified.

Dihydropyrimidine dehydrogenase has been purified from several sources. A dihydrouracil dehydrogenase has been purified from *Clostridium uracilium* by Campbell (24, 25). The purified enzyme is similar in many respects to dihydroorotate dehydrogenase originally described by Lieberman & Kornberg (123) in *Zymobacterium oroticum*. Both reactions are reversible and use DPN<sup>+</sup> as the H acceptor. Dihydrouracil dehydrogenase purified from *C. uracilium* does not act on either thymine or orotic acid. A dihydropyrimidine dehydrogenase has been purified about twenty-fold from beef liver acetone powders by Grisolia & Cardoso (67). The enzyme is highly unstable, and in contrast to the bacterial enzyme, the animal enzyme is specific for TPN as the hydrogen acceptor in agreement with earlier findings of Canellakis (26). Uracil and thymine as well as 5-bromouracil and 5-iodouracil can act as substrates. The reaction was studied in the direction of pyrimidine synthesis from dihydropyrimidine by measuring TPNH formation.

Dihydropyrimidine hydrolase, the enzyme catalyzing the hydrolysis of the dihydropyrimidines to carbamyl amino acids, has been demonstrated in soluble preparations of calf, rat, and pigeon livers by Grisolia & Wallach (68, 196). The enzyme catalyzing the hydrolysis of dihydrothymine to carbamyl- $\beta$ -aminoisobutyric acid ( $\beta$ -ureido isobutyric acid) has been purified two hundredfold from beef liver acetone powders, and found to be about 80 per cent pure by electrophoretic and sedimentation analysis (196). The reaction is reversible, but is pH dependent. The apparent equilibrium constant in the direction of ring closure is 1.5 at pH 5.0, the optimum pH value. The optimum pH for ring opening is 8.5. The purified enzyme also hydrolyzes hydantoin to carbamylglycine. However, this reaction could not be reversed. The  $K_m$  values obtained for hydantoin, hydrouracil, and hydrothymine are  $8.3 \times 10^{-4}$ ,  $11.75 \times 10^{-3}$  and  $2.1 \times 10^{-3}$  respectively.

Despite the demonstrated reversibility of the enzymatic reactions resulting in the conversion of uracil to carbamyl- $\beta$ -alanine and of thymine to carbamyl- $\beta$ -aminoisobutyric acid, the effectiveness of these reactions in the net synthesis of uracil and thymine from  $\beta$ -amino acids or the carbamyl- $\beta$ -amino acids in an *in vivo* system has yet to be demonstrated.

An enzyme catalyzing the formation of uridylic acid from uracil and 5-phosphoribosylpyrophosphate has been demonstrated in sonic extracts of several bacterial species (27, 37). This enzyme is analogous to the orotidine-5'-phosphate pyrophosphorylase first described by Lieberman *et al.* (124) in yeast and livers of several animal species. The uridine-5'-phosphate pyrophosphorylase has been found in various lactic acid bacteria which can utilize uracil for growth [Crawford, *et al.* (37)]. The enzyme is present in *Lactobacillus bulgaricus* 09X, a uracil requiring mutant of the parent strain, *L. bulgaricus* 09 (27). However, the parent strain, which utilizes orotic acid



but not uracil for growth, and forms uridylic acid from orotic acid but not from uracil, is apparently missing the uridine-5'-phosphate pyrophosphorylase (37). Uridine-5'-phosphate pyrophosphorylase has been purified from *Lactobacillus bifidus* and shown to differ from orotidine-5'-phosphate pyrophosphorylase (37).

Although uridine-5'-phosphate pyrophosphorylase was not found in rat liver, an enzymatic reaction effective in the synthesis of uridine-5'-phosphate from uracil could be demonstrated [Canellakis (27)]. A nucleotide phosphorylase which catalyzes the reversible phosphorolysis of uridine to uracil and ribose-1-phosphate was purified about ten-fold from rat liver acetone powders (27). Cytidine was not acted upon by this enzyme preparation. A uridine kinase was also partially purified from this rat liver acetone powder. The products of the enzymatic reaction between uridine and various nucleotide di- and triphosphates were isolated and identified as the mono-, di- and triphosphates of uridine (27).

It has thus been demonstrated that uridine-5'-phosphate can be formed by several distinct enzymatic pathways, and the biosynthetic mechanism involved in a particular biological system under particular conditions merits attention. It has, in fact, been found that when relatively high concentrations of the compounds are used, orotic acid, uridine-5'-phosphate and uracil are incorporated into RNA by rat liver slices at comparable rates [Canellakis (28)]. Slices of tissues with a more limited capacity to degrade uracil were found to incorporate relatively more uracil into RNA than those tissues with a greater capacity to degrade the base. It is therefore pointed out by Canellakis (28) that the metabolic fate of uracil depends on the effectiveness of two competing pathways: one leading to the synthesis of uridine-5'-phosphate and RNA formation and the other to the formation of dihydrouracil and finally degradation to carbon dioxide.

Certain *E. coli* mutants, which were originally classified as pyrimidineless mutants because of their response to dihydrouracil, have been found to be pantothenic acid mutants (189). It has been shown that dihydrouracil is not converted to RNA by the organisms, but is apparently degraded to carbamyl- $\beta$ -alanine and finally to  $\beta$ -alanine, which can be used for growth as well as pantothenic acid.

#### THYMINE

A general approach to the enzymatic mechanism of thymine biosynthesis was provided by Friedkin & Roberts (55), who demonstrated the conversion of deoxyuridine to thymidine using chick embryo and rabbit or chick bone marrow preparations. During the past year several soluble preparations which catalyze related reactions have been obtained. One of these, derived from *E. coli*, converts deoxyuridylic acid to thymidylic acid [Friedkin (54)]. After treatment with Dowex-1-formate, the extracts must be supplemented with tetrahydrofolic acid, ATP,  $Mg^{++}$ , and either serine or formaldehyde. A soluble preparation, described by Phear & Greenberg (160), has been ob-



tained from rat thymus gland and forms a mixture of thymidine and thymidylic acid from deoxyuridine. After treatment with Dowex-1-chloride, thymidine formation is stimulated by the addition of DPN, DPNH or TPNH and the other cofactors of the *E. coli* system. Another soluble preparation which converts uridine deoxyriboside to thymidine has been obtained from rabbit thymus homogenate by Blakley (15). Requirements for ATP, tetrahydrofolic acid, and DPNH could be demonstrated after Dowex treatment. Serine was a more active one-carbon donor than either formate or formaldehyde. Uridine, dihydrouracil, and dihydrouracil deoxyriboside were less active as substrates than uracil deoxyriboside. The significance of the observation that uracil deoxyriboside is converted to thymidine much more readily than deoxyuridylic acid is converted to thymidylic acid is difficult to interpret without a more detailed description of the experiment.

Thymidine has also been obtained as the product of the deamination of the relatively rare pyrimidine derivative, 5-methyldeoxycytidine by Cohen & Barner (33) using a partially purified preparation of "deoxycytidine deaminase" prepared from *E. coli* B. This enzyme, which deaminates deoxycytidine 2.5 times faster than cytidine, has no activity on 5-methylcytosine. A thymine-requiring strain of *E. coli* could utilize 5-methyldeoxycytidine but not 5-methylcytosine for growth.

Formaldehyde, formic acid, and serine have been shown to serve as sources of the one-carbon moiety of the thymine methyl group. The methyl group of methionine does not serve in this capacity (60). Two general mechanisms for the enzymatic reduction of these one-carbon sources to the methyl group of thymine have been advanced. Green & Cohen have suggested a series of reactions involving the dihydrohydroxymethyl pyrimidines as obligatory intermediates (60). According to this theory, the reduction of the hydroxymethyl group to the methyl group is accompanied by the oxidation of the dihydroheterocyclic ring to the pyrimidine ring. In order to test this hypothesis, dihydrocytosine, and its nucleoside and deoxynucleoside derivatives were synthesized (62), as well as dihydrothymine, dihydrouracil, and the nucleoside and deoxynucleosides of these compounds (61). No evidence could be obtained for the conversion of these compounds or of hydroxymethyluracil, hydroxymethylcytosine, or their deoxynucleosides to thymine or its derivatives in isotope competition experiments using various pyrimidineless mutants of *E. coli* (34), nor could the hydroxymethyl derivatives support growth of a thymine-requiring mutant (59).

The second theory, based on the observed requirements for tetrahydrofolic acid in the formation of the thymine derivative (15, 54, 160), relates the reduction of the hydroxymethyl group derived from serine, formate, or formaldehyde to the oxidation of the hydroxymethyl group carrier, tetrahydrofolic acid, to dihydrofolic acid (54, 160).

Flaks & Cohen (48a) have recently shown that the formation of 5-hydroxymethylcytosine takes place at the level of the nucleotide in cell-free extracts of *E. coli* infected with T6 bacteriophage. After treatment with

Dowex, the enzyme system required deoxycytidylate and tetrahydrofolic acid for the incorporation of formaldehyde- $C^{14}$  into acid-stable, nonvolatile form.

#### PYRIMIDINE ANALOGUES AND INHIBITORS

5-Fluorouracil and 5-fluoroorotic acid (44) markedly inhibit the growth of transplanted tumors in rats and mice (84). These compounds are also active as bacteriostatic agents (84, 178). Evidence was obtained for the incorporation into RNA of 5-fluorouracil administered to rats (82, 84), as well as complete inhibition of formate incorporation into thymine *in vitro* by the analogue. The deoxyriboside of 5-fluorouracil was shown to be much more effective than the riboside or the free base in inhibiting formate incorporation into thymine *in vitro* (83). It is suggested that this analogue is converted to the deoxynucleotide and exerts its primary effect by inhibiting the conversion of deoxyuridylic acid to thymidylic acid (45, 83). The synthesis of 2-fluoroadenosine and its activity in inhibiting growth of a human carcinoma has been reported (147).

In studies on the bacteriocidal action of 6-azauracil (*as*-triazine-3,5-dione), it was found that a resistant strain of *S. faecalis* has lost the ability to convert the 6-azauracil to the riboside derivative and failed to incorporate uracil into its RNA (76). These findings suggest that azauracil must first be converted to a riboside derivative in order to exert bacteriostatic action. Similar conclusions were reached on the basis of experiments with mammalian tissue cultures (179).

6-Azathymine is also converted to the deoxyriboside and is incorporated into the DNA of *S. faecalis* (166). Evidence for the incorporation of thiouracil into the RNA of tobacco mosaic virus has been confirmed (101, 136). It has been suggested that these analogues exert their inhibitory action after incorporation into a nonfunctional nucleic acid. It is also possible that the primary effect of the analogue is on a specific enzymatic reaction involved in pyrimidine metabolism which has not yet been demonstrated.

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## METABOLIC ANTAGONISTS<sup>1,2</sup>

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### STRUCTURE AND BIOLOGICAL ANTAGONISM

It has been known for some time that the structural features necessary for a metabolite antagonist include certain groups necessary for complexing with an enzyme and also certain specific distances between such groups (1). Recently, much attention has been devoted to structural considerations other than the active groups and to other effects, such as hydration of groups which are involved in binding at the active sites. The relationships of structure and biological activity of recently reported metabolite analogues of general metabolic significance are included in this section.

*Amino acid antagonists.*—An inhibitory effect on L-glutamic acid dehydrogenase is observed not only with isophthalic acid, but also with *m*-nitro- and halobenzoates. A dipole interaction allows binding of these latter groups analogous to that of carboxyl groups. On the basis of charge interaction, the carboxyl group would bind more effectively; however, the equilibrium constants for isophthalate and *m*-bromobenzoate are approximately equal. These results are explained by the greater degree of hydration of the isophthalate, resulting in an opposing effect which accounts for the similar activity (2).

Specificity of configuration of groups on carbon atoms other than those binding functional groups may also be essential for an antagonist. For example, O-methylthreonine, but not O-methylallothreonine, is a competitive antagonist of isoleucine incorporation into the proteins of ascites cells, indicating that the O-methyl group of the inhibitor is in the same steric position as the ethyl group in isoleucine (3). Cyclopentaneglycine specifically inhibits the utilization of isoleucine in *Escherichia coli* (4), while the toxicity of 2-cyclopenteneglycine is reversed competitively by a mixture of isoleucine and valine but not by either alone (5). This difference in specificity is attributed to slight puckering of the cyclopentane ring and the more planar structure of the cyclopentene ring which permits only the latter group to occupy the position of the planar isopropyl group of valine in enzyme combinations,

<sup>1</sup> The survey of literature pertaining to this review was completed in October, 1957.

<sup>2</sup> The following abbreviations are used in this chapter: ATP for adenosine triphosphate; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; DPNase for diphosphopyridine nucleotidase; RNA for ribonucleic acid; and TPN for triphosphopyridine nucleotide.

<sup>3</sup> The authors are indebted to Dr. R. G. Ham for preparing the section on "Antimetabolites in the Study of Development and Differentiation," and to Dr. E. M. Lansford, Jr., for assistance with the manuscript and for preparing the section on "Antimetabolites in Tumor and Other Chemotherapy."

while both analogues are capable of antagonizing isoleucine which contains a *sec*-butyl group in the corresponding position. 2-Cyclohexene-1-glycine is also an antagonist of isoleucine in *E. coli*, but cyclohexaneglycine is not; since both compounds can theoretically exist in similar boatlike structures, it appears that the inhibitory form of the unsaturated analogue does not possess this structure, but rather one in which carbons 5 and 6 are on opposite side of the plane containing carbons 1, 2, 3, and 4 (6). In *Leuconostoc dextranicum* 8086, cyclopentanealanine competitively inhibits the utilization of leucine but not phenylalanine, whereas 1-cyclopentenealanine inhibits the utilization of phenylalanine but not leucine (7). 1-Cyclohexenealanine also inhibits phenylalanine utilization, and cyclohexanealanine does not (6).

The emanation of the first carbon of the side chain in the plane of the 1-carbon and two adjacent carbons of the ring appears to be essential for phenylalanine antagonism. The cyclopentyl group (but not cyclohexyl) is sufficiently similar to the isopropyl group of leucine to allow antagonism of leucine by cyclopentanealanine. The side chain of the latter compound does not emanate in the plane of the ring as does the side chain of 1-cyclopentenealanine.

The replacement of a  $-CH_3$  group by an  $-O-$  atom, successful in producing an isoleucine antagonist as indicated above, also produces a competitive glutamine antagonist, O-carbamyl-L-serine (8), effective in several microorganisms. Azaserine (O-diazoacetyl-L-serine) (9) is, under certain conditions, an antagonist of glutamine (p. 664).

The introduction of a nitrogen in lieu of a carbon atom in the aromatic group of a metabolite has resulted frequently in an inhibitory analogue. Recent applications of this structural change in amino acids include 1,2,4-triazole-3-alanine (azahistidine), a specific competitive antagonist of histidine in certain strains of *E. coli* (10); 7-azatryptophan (11), an antagonist of tryptophan in *Tetrahymena pyriformis* (11) but not in a mutant strain of *Neurospora crassa* requiring tryptophan or indole for growth (12); 7-azaindole (13), an inhibitor of the utilization of indole in the *Neurospora* mutant (12); and tryptazan (2-azatryptophan), a competitive antagonist of tryptophan utilization in yeast (14).

A number of sulfur-containing amino acid analogues have recently been prepared. 3-Amino-3-carboxypropanesulfonamide which contains a sulfonamide group, in lieu of the carboxamide group of glutamine inhibits the growth of *E. coli* and suppresses multiplication of  $T_2$  coliphage, and its toxicity is reversed by glutamine or glutamic acid; however, it is inactive in mice infected with *E. coli* (15). 2-Thiohydantoin-5-acetic acid inhibits growth of *Lactobacillus casei*, and the inhibition is almost completely reversed by either asparagine or aspartic acid (16). The effect of S-alkylhomocysteines on the growth of *Salmonella enteritidis*, *E. coli*, and their methionine-requiring mutants shows that the inhibitory effect on methionine utilization decreases with increasing size of the alkyl group in the series: ethyl, propyl, butyl, and isoamyl (17).

Halogenated amino acids, particularly fluoroderivatives, are frequently effective amino acid antagonists. *p*-Fluorophenylserine is quite similar to phenylserine as a phenylalanine antagonist of *E. coli* (18); however, the corresponding chloro derivatives are relatively inactive as phenylalanine antagonists (19). 4- and 5-Fluoroanthranilic acid inhibit *E. coli*, and the inhibition is reversed by anthranilic acid, indole, tryptophan and also by other amino acids including tyrosine; 5-methylantranilic acid yields comparable results (20). Of a group of  $\omega$ -trifluoromethyl amino acids, the norvaline analog inhibits *E. coli*; methionine, leucine, and, less effectively, valine, reverse the growth inhibition (21).

The introduction of an  $\alpha$ -methyl group in an amino acid, e.g.,  $\alpha$ -methylglutamic acid whose biological properties have recently been reviewed (22), has produced a few antimetabolites.  $\alpha$ -Methylmethionine is reported to be a methionine antagonist (23). Another analogue,  $\alpha$ -methyl-valine, as well as S-ethylcysteine, inhibits penicillin synthesis from lactose, and the inhibitory effects are prevented by valine and cysteine, respectively (24).

Acetylenic amino acid analogs such as propargylglycine and 2-amino-3-methyl-4-pentynoic acid are more inhibitory than the corresponding ethylenic derivatives for yeast but not for *E. coli* (25). Propargylglycine inhibition of growth of *E. coli* is reversed by leucine or valine; however, for yeast and rats, complete reversal of toxicity was not observed even with mixtures of amino acids (26).

*m*-Aminophenylalanine is reported to be a phenylalanine antagonist for *E. coli*, but for *Saccharomyces cerevisiae*, it is reported to be a competitive antagonist of lysine (26).

Ethyl diazopyruvate suppresses the growth of *E. coli*, and the toxicity is reversed by leucine and isoleucine, and to a lesser extent by other amino acids (27).

*Purine and pyrimidine antagonists.*—The inhibitory effect of certain purine and pyrimidine analogues on growth of tumors has stimulated the synthesis of a large number of these compounds. Among the new analogues of interest are some fluoro derivatives which are quite inhibitory to a number of transplanted tumors and microorganisms. These compounds include 2-fluoroadenosine (28), 5-fluorouracil, 5-fluorocytosine and 5-fluoroorotic acid (29). Complete biochemical studies have not as yet been reported on these derivatives (p. 655).

The replacement of a carbon atom by a nitrogen atom, an approach which was successful in producing inhibitory analogues of purines, has also produced inhibitory pyrimidine analogues. A thymidine antagonist, azathymidine (the deoxyriboside of 6-azathymine) is more active than the parent pyrimidine as an inhibitor of growth of several bacteria (30). An improved method of synthesis of 6-azauracil has been reported (31), and its competitive reversal by uracil, cytosine, and uridine in several microorganisms (32) was observed.

Substitution of a group in the 5-position of natural pyrimidines has fre-

quently resulted in active pyrimidine antagonists. New pyrimidines of this type include 5-mercaptouracil, 5-uracilyl disulfide, and uracil-5-isothio-uronium chloride, which inhibit the utilization of thymine in *L. leichmanii*; thymidine reverses this inhibition noncompetitively (33). 5-Hydroxy- and 5-bromodeoxyuridine suppress the growth of *E. coli* (34). The 5-hydroxy analogue competitively inhibits the utilization of uridine and less effectively deoxyuridine in *E. coli* (35), while the bromo analogue is an excellent competitive inhibitor of thymidine utilization in *Lactobacillus arabinosus* (33). 5-Aminodeoxyuridine also inhibits the utilization of deoxyuridine in *E. coli*, and thymidine reverses the inhibition noncompetitively (35). 5-Chloro-, 5-bromo- and 5-aminocytidine are more effective than the corresponding uridine derivatives in inhibiting the utilization of uridine and cytidine in *Neurospora* 1298, and the analogues inhibit more effectively growth stimulation by cytidine than by uridine (36).

Of a group of substituted pyrimidines, 2,4-diamino-6-hydroxy-5-nitroso- and 4-amino-6-hydroxy-2-thiomethylpyrimidine inhibit growth of a purine-less strain of *E. coli* with reversal by either adenine or guanine (37). 5-Iodo-2-thiouracil and 5-iodo-2-benzylthiouracil inhibit the growth of *S. faecalis*, though not as effectively as the well-known inhibitors, 5-amino-, 5-nitro- and 2-thiouracil, with reversal by either uracil or thymine, except for 2-thiouracil which is reversed by uracil alone (38). 2-Amino- and 2-acylaminopyrimidines and their 4-methyl- and 4,6-dimethyl-homologues are lethal for *Endamoeba histolytica*; these toxicities are reversed by a mixture of uracil, cytosine, and thymine (39). The inhibition of puromycin and its nucleoside fragment on the latter organism is reversed by adenylic acid but not by adenine (39); however, against an experimental *T. equiperdum* infection in mice these derivatives were reversed by adenine and a number of related compounds (40).

6-Uracilsulfonic acid, 6-uracilsulfonamide, and 6-uracil methyl sulfone inhibit the conversion of orotic acid to orotidine-5'-phosphate by a partially purified pyrophosphorylase of yeast (41), but these compounds are ineffective as inhibitors of a number of microorganisms (42).

A large number of potential purine antagonists consisting of substituted pyrazolo(3,4-d)pyrimidines which have anti-tumor activity have recently been prepared (43, 44). In studies with several strains of *N. crassa*, 5 of 25 analogs tested were toxic, and the most efficient inhibitor was 4-amino-pyrazolo(3,4-d)pyrimidine. Its toxicity was reversed by adenine, pyridoxine, thiamine, caffeine, and less effectively by some derivatives with the pyrazolo(3,4-d)pyrimidine nucleus (45).

Replacement of a nitrogen by a carbon atom has resulted in deazapurines which have some ability to antagonize purines. 1-Deazaguanine is a weak guanine antagonist for *Tetrahymena pyriformis*, but 1-deazaadenine is effective as an antagonist of adenine for this organism (46).

An aza analogue, 4-aminotriazole-5-carboxamide, is an antagonist of the incorporation of aminoimidazolecarboxamide into polynucleotides of rat or pigeon liver slices (47).

*Vitamin analogues.*—The continued interest in folic acid antagonists as antitumor agents has stimulated the preparation of a large number of pteridines and related compounds. Of these, only a few have actually shown antagonism of folic acid or related derivatives, and even fewer are comparable in activity to those prepared some years ago. Some recent syntheses have involved alterations in the *p*-aminobenzoic acid moiety of the parent structure. For example, N-(2-amino-4-hydroxy-6-pteridyl)methyl substituted derivatives of *o*-, *m*- and *p*-toluidine (48), *p*-aminomethylbenzoic acid (49) and arsanilic acid (50), and in the last case also with a corresponding 2,4-diamino substituent, are growth inhibitors of which all except the 2,4-diamino derivative of arsanilic acid are reversed effectively by folic acid in *S. faecalis*. An O-(2-amino-4-hydroxy- and 2,4-diamino-6-pteridyl)methyl substituent on *p*-anistic acid and its conjugate containing glutamic acid produced some oxygen analogues of folic acid which are inhibitory for *S. faecalis*, and their toxicities, with the exception of the 2,4-diaminoanalogue, are reversed by folic acid (51). The introduction of *p*-aminosalicylic acid in place of the *p*-aminobenzoic acid moiety of folic acid produces an analogue of moderate activity for *S. faecalis* (52). The analogue containing sulfanilic acid in lieu of the *p*-aminobenzoic acid moiety of folic acid has been known for some time, but it has recently been shown that glycine may replace the glutamic acid group in this derivative with retention of anti-folic acid activity (53).

Two new types of folic acid antagonists for *S. faecalis*, 2,4-diamino-5-arylazopyrimidines and 2,6-diamino-8-aryl-8-azapurines, have been described (54, 55). Both the diamino and 8-aryl group, which may contain a halogen substituent, appear to be essential for activity in the azapurine series. The diaminoaryl azopyrimidine derivatives containing a 6-amino group are usually effective, and this group can be replaced by a hydroxy or mercapto group or hydrogen and still retain activity. While the arylazopyrimidines can be chemically converted to the azapurines, this does not appear to account for their biological activity. The toxicity of one of the analogues, 2,4-diamino-6-hydroxy-5-phenylazopyrimidine, though reversed by folic acid in *S. faecalis*, is prevented in an *E. coli* mutant by purines (55). It is possible that purines may be secondary reversing agents for *E. coli*; this organism does not utilize folic acid.

The reduction of the pteridine nucleus in an attempt to obtain a more effective antagonist corresponding to folinic acid has not been very successful; however, dihydro-2-desaminofolic acid, but not tetrahydro-4-amino-4-deoxyfolic acid, inhibits the synthesis of serine from glycine and formaldehyde by a partially purified enzyme from rabbit liver (56).

Substituted pteridines have received considerable attention, and one of the more interesting is 2,4-diamino-6,7-(4',5'-dimethylbenzo)pteridine, the toxicity of which for *L. leichmannii*, at low concentrations, is reversed by folinic acid but not by folic acid; whereas, at high concentration, both riboflavin and folinic acid are required for reversal; thus this compound appears to antagonize two structurally related vitamins (57). Some 6,7-disubsti-

tuted-2,4-diaminopteridines are effective antagonists of folic acid in *Leuconostoc citrovorum*, and the diisopropyl and di-*sec*-butyl derivatives are the more active (58). 7-Amino and 7-hydroxy substituted 6-aryl-2,4-diaminopteridines possess some anti-folic activity, but the most active, such as 2,4,7-triamino-6-*p*-chlorophenylpteridine, are only one-hundredth as effective as 2,4-diamino-6,7-diphenylpteridine (59). In a study of quinoline derivatives, the toxicity of N-(2-diethylaminoethyl)-(7-chloro-3-methyl-quinolyl-4)-mercaptoacetamide for *S. faecalis* was reversed by folic acid (60).

A thioamide group in place of the carboxyl group of some *p*-aminobenzoic acid derivatives has resulted in antagonists with antibacterial power similar to sulfanilamide for *Micrococcus pyogenes* var. *aureus* and *E. coli*; active compounds include *p*-aminothiobenzamide and its phenyl and 3,4-dimethyl-5-isoxazolyl substituted amides (61).

Other than the benzopteridine compounds which antagonize both riboflavin and folic acid, analogues with either 9-formyl, 9-(2'-hydroxyethyl), or 9-(2'-acetoxyethyl) groups replacing the 9-(D,1'-ribityl) group of riboflavin are riboflavin antagonists in the rat, and in the case of the latter two, in *L. casei* as well (62). Early studies demonstrated that 6-ethyl-7-methyl-9-(D,1'-ribityl) isoalloxazine effectively replaces riboflavin in promoting growth of *L. casei*, and recently the other isomer, the 6-methyl-7-ethyl derivative, was shown to possess similar activity (63). In *L. casei*, riboflavin monosulfate inhibits the utilization of riboflavin, its mononucleotide and dinucleotide, with inhibition indices for half-maximal growth of 63, 6 and 5, respectively (64). Riboflavin monosulfate and adenosine-5'-monosulfate both inhibit the action of D-amino acid oxidase on D-alanine with flavin adenine dinucleotide; each inhibitor reacts independently with the enzyme and both increase their respective affinities for the enzyme (65).

Derivatives of 4-thiazolidone-2-caproic acid (acidomycin), a biotin antagonist for *Mycobacterium avium*, such as its methyl ester, hydrazide, and the corresponding alcohol similarly antagonize biotin (66). The bishydrazides of pimelic, suberic and sebacic acids, as well as the hydrazide of 2-imidazolidinone-4-valeric acid, similar to the corresponding caproic acid derivative, also exert antimicrobial effects which are reversed by biotin (67).

Of a number of lipoic acid analogues studied, 5-methyl-1,2-dithiolane-3-valeric acid (8-methylthiotic acid), a weak growth-promoting agent for *Corynebacterium bovis*, is a competitive inhibitor of lipoic acid for *S. faecalis* and *Tetrahymena geleii* (68). 6-Alkyl-8-thiooctanoic acids inhibit *Torula cremoris*, but these inhibitions are not reversed by lipoic acid (69). One of these analogues, 6-ethyl-8-thiooctanoic acid, inhibits pyruvate oxidation, and some evidence was obtained indicating this compound blocks the formation of 6-acetylthio-8-thiooctanoic acid. The compound also interferes markedly with phosphotransacetylase activity under certain conditions (70).

The pyrimidine moiety of thiamin and the vitamin B<sub>6</sub> group show some



mutual antagonisms (see p. 663). Replacement of the 2-methyl group of pyridoxine by 2-ethyl was an early modification of the vitamin, and recently, the synthesis and biological activities of the corresponding homologues of pyridoxal and pyridoxamine, and their phosphorylated derivatives, have been reported. The unphosphorylated compounds show growth promoting activity for lactic acid bacteria but act as vitamin B<sub>6</sub> antagonists for yeast (71). See p. 658 for related biological activities of these compounds.

Replacement of the methyl group of the pyrimidine moiety of thiamine by a methylthio group results in an inhibitor of thiamine utilization for a mutant strain of *E. coli* (72). The use of a combination of modifications, each of which had previously produced thiamine antagonists, has been attempted. Hydroxy substitution for the amino group of pyrithiamine and butylthiamine does not result in effective inhibitors; however, butylpyrithiamine is an effective antagonist of thiamine in pigeons (73).

6-Aminonicotinamide is a potent nicotinamide antagonist (74), whereas the corresponding acid had been found previously to be an antagonist of both nicotinic acid and *p*-aminobenzoic acid.

$\beta$ -D-Pantoylaminoethanethiol and the corresponding disulfide antagonize the function of pantothenic acid *in vivo* for the rat (75). This analogue also inhibits noncompetitively the synthesis of coenzyme A from pantetheine *in vitro*, and competitively inhibits coenzyme A functions (75). In *E. histolytica*, a reversal of inhibition by the disulfide analogue occurs with pantetheine and coenzyme A, but not with pantothenic acid (76).  $\omega$ -Methylpantetheine interferes with the utilization of pantetheine in *Lactobacillus helveticus* (77). An oxygen analogue of pantetheine (N-pantothenylethanolamine) is a competitive inhibitor of pantetheine in *L. helveticus* (78). Another analogue of pantothenic acid, N-( $\alpha,\gamma$ -dihydroxy- $\beta,\beta$ -diethylbutyryl)- $\beta$ -alanine, has growth promoting effects on two organisms rather than growth-inhibiting action (79).

#### BIOLOGICAL STUDIES OF METABOLITE ANTAGONISTS

Inhibitors are too frequently termed metabolite antagonists solely on the basis of structural similarity and some degree of metabolite reversal at the lowest effective concentration of the inhibitor. Competitive reversal of an inhibition with a high degree of specificity by the metabolite over a broad range of concentrations as is feasible in the biological system should be demonstrated before using an analogue as a competitive metabolite antagonist in biochemical studies. Studies with a competitive antagonist should be attempted only under conditions such that the inhibition is the limiting process and that nutritional and other factors do not appreciably limit the response in the absence of the inhibitor. A deficiency of a growth factor and an inhibition of its utilization are not equivalent since the latter frequently affects fewer processes or affects more strongly a particular enzyme system.

For those inhibitions involving competition of an analogue with a metabolite for an enzyme site, an analysis of the effect of reversing agents other

than the metabolite can, if properly done, give valid results in demonstrating metabolic pathways in living organisms. A secondary reversing agent can theoretically affect a competitive system in four ways: (a) it may alter the intracellular concentrations of the competing analogue and substrate, or compete per se at the enzyme site; (b) it may replace the product, so that inhibition of the particular enzyme is of no consequence to the biological system; (c) it may act by sparing the amount of product needed; or (d) it may increase the relative number of effective enzyme sites. Methods have been reported previously for distinguishing each general type of secondary reversing agent from others. The magnitude of the effect of a secondary reversing agent is important in interpreting the inhibition analysis data since small nonspecific effects are frequently of no consequence and quite removed from the specific system.

*Amino acid interrelationships.*—Analyses of cysteic acid inhibition of lactobacilli have demonstrated essential roles of aspartic acid in the biosynthesis of lysine, of threonine through homoserine (or a derivative), of uracil, and of purines at a stage prior to aminoimidazolecarboxamide (80, 81, 82). These data were obtained prior to, or independently of, comparable demonstrations by other techniques. Glutamic acid similarly inhibits aspartic acid utilization preventing certain of these processes, and inhibits synergistically with cysteic acid (83, 84). Independent and essential roles of glutamic acid in the conversion of aspartic acid to pyrimidines and in the ornithine-citrulline conversion were demonstrated with aspartic acid and arginine as inhibitors of glutamic utilization in certain lactobacilli (85, 86). This gave early evidence that the incorporation of the carbamyl group into these products occurs by different routes, rather than by a single route through citrulline to arginosuccinic acid and then to pyrimidines. An essential role of glutamic acid in purine biosynthesis was similarly demonstrated.

Some enzyme studies have shown that  $\beta$ -hydroxyaspartic acid, an aspartic acid analogue inhibitory in many organisms, is formed by transamination of dihydroxyfumaric acid and glutamic acid (87), and the enzymatic transamination of aspartic acid is inhibited competitively by  $\beta$ -hydroxyaspartic acid and by 2,3-diaminosuccinic acid which also has a noncompetitive inhibitory effect analogous to 2,3-diaminopropionic acid (88). Canavanine, an inhibitory analogue of arginine, and fumarate are converted to canavansuccinic acid in acetone-dried hog kidney preparations (89).  $\alpha$ -Methylaspartic acid appears to exert an antimetabolite action in inhibiting the conversion of aspartic acid and citrulline to arginosuccinic acid by rat liver sections (90), and  $\alpha$ -methylglutamic acid inhibits glutamic-pyruvic transaminase (91). D-Aspartic acid inhibits the oxidation of L-aspartic acid in *Shigella flexneri* (92).

Detection and assay of the methylsulfonium derivative of methionine during its isolation from cabbage juice was accomplished using sulfonamide-inhibited *E. coli*, for which this derivative is a more effective reversing agent than methionine (93). The ethyl sulfonium derivative of ethionine competi-

tively inhibits the utilization of the methyl-sulfonium derivative in this assay; methionine reverses the toxicity over a range of concentrations but not at high inhibitor concentrations which are reversed by the methyl sulfonium derivative. These results gave an early indication of the transfer of a methyl group to form methionine and suggested additional essential functions of the compound in other methylation reactions (94). Ethionine inhibition of *Ochromonas malhamensis* is reversed by methionine, or by single carbon unit precursors such as serine and glycine, only if vitamin B<sub>12</sub> is present (95). Aromatic amino acids also exert a reversing effect on methionine antagonists (96).

Sometimes two or more metabolites are found which competitively reverse the toxicity of an analogue over a range of concentration. For example, arginine, homoarginine, or lysine reverse in this manner canavanine inhibition of growth of *Torulopsis utilis*; and for *Chlorella vulgaris*, the toxicity of both canavanine and homoarginine is reversed competitively by either lysine or arginine (97, 98). The analogue in such instances apparently must be acted upon before inhibiting an endogenous metabolite, and substances capable of preventing this action upon the analogue reverse the inhibition. In *E. coli* B, inhibition of growth by homoarginine is reversed competitively by arginine over a range of concentrations, but high concentrations of arginine reverse noncompetitively. The possibility that homoarginine inhibits a function of arginine in its own biosynthesis was considered (99).

Uniformly labeled tyrosine reverses phenylserine inhibition of the utilization of phenylalanine in *E. coli* as a type (a) reversing agent but is not incorporated into cellular phenylalanine under these conditions; it has been proposed that tyrosine, in preventing its own biosynthesis, diverts the common precursor of both amino acids into phenylalanine (100).

Inhibition of growth of certain microorganisms by 4- or 5-methyltryptophan is reversed noncompetitively by tryptophan and indole (101, 102), while anthranilate competitively reverses the toxicity of the latter compound for *Lactobacillus plantarum*, and phenylalanine exerts a reversing effect for certain lactobacilli (101). For *E. coli*, 4-methyltryptophan inhibits tryptophan desmolase, the synthesis of anthranilate, and indole utilization, but does not affect the conversion of anthranilate to indole (102).

An interesting use of a metabolite analogue in determining the course of a biosynthetic process is the conversion of 4-methylanthranilic acid to 6-methylindole in an *E. coli* mutant requiring tryptophan, indicating that the pyrrole ring is closed at the position of the carboxyl group in anthranilic acid (103). Tryptophanase of *E. coli* degrades 4- and 5-methyltryptophan (102, 104). 5-Hydroxytryptophan, though not degraded itself, inhibits degradation of tryptophan by rabbit liver, but not by *E. coli* (104).

*Amino acid activation.*—The recently discovered activation of amino acids by specific enzymatic interactions with adenosine triphosphate has been extended to amino acid analogues. The pancreas tryptophan enzyme activates tryptozan (2-azatryptophan), 7-azatryptophan and 5- and 6-fluoro-

tryptophan in the same manner as tryptophan; however,  $\beta$ -methyltryptophan, tryptophan hydroxamate, tryptamine, 5-hydroxytryptophan, 5- and 6-methyltryptophan, and D-tryptophan do not undergo the activation but do inhibit the utilization of tryptophan by the enzyme (105). A correlation between the ability of an analogue to undergo the activation reaction and its incorporation into protein was shown (105).

*Amino acid transport.*—The concentration in cells of nutritional substances at a higher level than the exogenous supply has recently been investigated with antimetabolites. In *E. coli* (106, 107), individual amino acids are concentrated into the cells by specific energy requiring mechanisms. The concentrated intracellular amino acid rapidly equilibrates with external amino acid, or can be very rapidly displaced by certain of its analogues (106, 108). Isoleucine, leucine, norleucine, threonine, and, to some extent, methionine all displace valine concentrated in the cell; *p*-fluorophenylalanine displaces phenylalanine, and norleucine displaces methionine. A close relationship exists between the ratio of isoleucine to valine necessary for overcoming the growth inhibition by valine of a valine-sensitive strain of *E. coli*, and the ratio necessary for displacement of concentrated valine in the cell.

Similar results have been reported on the inhibition by a number of natural amino acids of histidine uptake into the mycelium of histidineless strains of *N. crassa* (109). In contrast to results with *E. coli*, histidine so concentrated and stored is not displaced at any appreciable rate by the exogenous inhibitors.

*Peptide and keto acid utilization.*—Enhanced activities of derivatives of amino acids over those of free amino acids have been known for some time. In several instances of enhanced peptide activity, the free amino acid was inhibited in its utilization by an antagonist (1). Keto acids and hydroxy acids are also capable of such action as illustrated by the reversal of natural antagonisms among isoleucine, valine and leucine by the keto acid of the inhibited amino acid in *L. dextranicum* (110), and by the inability of phenylalanine antagonists to inhibit the utilization of phenyllactic acid, or phenylpyruvic acid, in *L. casei* (111).

Inhibition studies with glycyl- $\beta$ -2-thienylalanine and  $\beta$ -2-thienylalanyl-glycine in *E. coli* show that the inhibitory effects of the two peptides are reversed in a competitive manner by the corresponding peptides of phenylalanine; however, noncompetitive reversal occurs with other peptides of phenylalanine and with phenylalanine. In contrast, leucylthienylalanine not only inhibits the utilization of its corresponding phenylalanine peptide, but other peptides of phenylalanine also (112). Thienylalanine does not effectively inhibit utilization of peptides of phenylalanine (112, 113); glycyl-glycylthienylalanine and thienylalanylglycylglycine inhibit only their corresponding peptide of phenylalanine (114).

A study of growth inhibitions by mixtures of  $\beta$ -2-thienylalanine, 2-thienylpyruvic acid and glycyl- $\beta$ -2-thienylalanine and reversals of the inhibitions by phenylalanine, phenylpyruvic acid, and glycylphenylalanine shows

that the amino acids, the keto acids and the peptides each have separate competitive sites of action which are not involved in the utilization of the other two (115). However, in *L. casei*, *m*-hydroxyphenylpyruvic acid inhibits growth stimulated by phenylalanine, its keto acid or the corresponding hydroxy acid, but phenylalanine analogues do not inhibit the utilization of phenylpyruvic acid (111).

Separate exclusive sites of utilization of an amino acid, its keto acid and peptides in the synthesis of the adaptive malic enzyme of *L. arabinosus* are demonstrated by the specificity of thienylalanine, its keto acid or peptides in inhibiting only the corresponding natural metabolite, phenylalanine or its derivatives; and are shown also by the specificity of isoleucine, its keto acid or glycylpeptide in inhibiting only the corresponding metabolites, valine or its derivatives (114, 116, 117). Cysteic acid and  $\beta$ -hydroxyaspartic acid inhibit synergistically the utilization of aspartic acid for malic enzyme synthesis suggesting inhibition of amino acid utilization at different stages by the inhibitors, neither of which inhibits the utilization of glycylasparagine in place of aspartic acid for enzyme synthesis (118).

It is apparent that exogenous amino acid utilization requires certain enzymatic or enzymatic-like interactions before reaching a common point in the utilization of peptides. The conversions of peptide and keto acid to an activated form or complex of the amino acid by separate routes, not involving an essential step in the utilization of the free amino acid, was suggested as the most logical explanation of the data; however, competitive analogue inhibition of cellular penetration of amino acids, without inhibition of penetration of peptides subsequently hydrolyzed intracellularly by the organism to circumvent the inhibition, has also been proposed. The primary difference in these proposals is whether or not the first common intermediate is free intracellular amino acid or an activated form (or complex) of the amino acid. An amino acid concentrated from the medium into the cells of *E. coli* is rapidly displaced by an exogenous analogue, or is rapidly equilibrated with exogenous labeled amino acid, but not with its peptide or keto acid. Since the keto acid and peptides can circumvent the inhibitory effect of the analogue, it appears that the common point in their utilization does not involve this form of intracellular amino acid. Recent evidence also indicates that this type of concentration with other substances occurs in protoplasts of *E. coli* (119), and one metabolite is concentrated in cells to such an extent that its solubility in water is exceeded (120).

A general method of demonstrating specific inhibition of exogenous amino acid utilization but not of biosynthetic amino acid involves peptide analogues. For example, phenylalanine reversal of growth inhibition of *E. coli* by glycylthienylalanine is prevented competitively by *p*-tolylalanine and  $\beta$ -1-naphthylalanine (114), neither of which alone affects growth; also, both reverse thienylalanine toxicity. The reversing effects of a number of methylated phenylalanines on thienylalanine inhibition could be the result

of this type of action (121). The above technique affords a method of demonstrating these effects.

*Protein and nucleic acid synthesis.*—Metabolite antagonists have been of importance in the study of nucleic acid and protein synthesis. It is now well known that analogues of purines and certain pyrimidines can not only inhibit RNA synthesis but in so doing also inhibit synthesis of proteins including adaptive and constitutive enzyme. Amino acid antimetabolites also inhibit both protein and RNA synthesis. Catalytic amounts of amino acids seem to be essential for RNA synthesis which apparently can proceed in the absence of protein synthesis (122). The close relationship of the biosyntheses of proteins and nucleic acids may involve common precursors (123), or they may be associated directly in their biosyntheses (124).

8-Azaguanine, even if added subsequent to induction of the adaptive enzymes, inhibits the formation of  $\beta$ -galactosidase and catalase by *Staphylococcus aureus*; but in a strain of the organism in which glucozymase and catalase are constitutive enzymes, the synthesis of these enzymes is not appreciably inhibited by 8-azaguanine in most stages of growth (125). Azaguanine does not prevent nucleic acid synthesis under these conditions but is incorporated into RNA. This analogue also inhibits amylase formation by a strain of *B. subtilis* (126). 2-Azaadenine inhibits both protein and RNA synthesis in a thymine-requiring mutant of *E. coli* with thymine withheld to restrict DNA synthesis. Thiouracil and 5-hydroxyuracil inhibit only in the early stages of synthesis (127).

Some specificity of action of analogues appears to exist in inhibiting synthesis of different enzymes. For example, 6-mercaptapurine, 2,6-diaminopurine, 5-aminouracil, 5-methyl-2-thiouracil, 6-methyl-2-thiouracil, 2-thiocytosine or 2-thiouracil all inhibit the formation of the adaptive enzymes for the oxidation of benzoic acid in *Mycobacterium tuberculosis*; however, only the first five analogues, and 2-thioorotic acid, which is ineffective in inhibiting the benzoic acid enzyme synthesis, inhibit the synthesis of the adaptive enzymes for the oxidation of myo-inositol (128).

Induced synthesis of maltase in yeast is inhibited by structural analogues of tryptophan, the most effective being tryptozan (2-azatryptophan) which also inhibits growth, enzyme synthesis and the utilization of the free amino acid pool (14).

Both methylglycine and  $\beta$ -benzoylalanine inhibit, and *p*-fluorophenylalanine partially inhibits, the synthesis of RNA, DNA and protein in *E. coli* B; whereas  $\beta$ -2-thienylalanine inhibits only protein and RNA synthesis but not DNA synthesis (129). The incorporation of various precursors into RNA by ribonucleoprotein particles isolated from pea seedlings is promoted by a mixture of amino acids, and inhibited by analogues such as *p*-fluorophenylalanine,  $\beta$ -2-thienylalanine, ethionine, allylglycine, and methionine sulfoxide (130). *p*-Fluorophenylalanine does not affect the incorporation of adenine and uracil into RNA during enzyme induction in resting yeast (131). 5-Methyltryptophan stops uracil-induced synthesis of protein without af-



fecting RNA synthesis in olive trees and grapevine branches, while thiouracil decreases the synthesis of both protein and RNA (132). Though protein is synthesized in *E. coli* in the presence of 4- or 5-methyltryptophan, or  $\beta$ -2-thienylalanine, synthesis of active  $\beta$ -galactosidase does not occur (133).

*p*-Fluorophenylalanine inhibits an increase of amylase activity in pigeon pancreas which requires supplements of ATP, arginine, threonine, and RNA (134). Ethionine strongly inhibits the synthesis of cellular proteins without inhibiting amylase formation by *B. subtilis* (135). Ethionine,  $\beta$ -aspartylhydrazide and  $\gamma$ -glutamylhydrazide all inhibit the formation of the adaptive enzyme for benzoic acid in *Micrococcus urae* (136).

$\beta$ -3-Thienylalanine or propargylglycine when fed to rats caused a loss of liver xanthine oxidase and prevented repletion of xanthine oxidase in protein-depleted rats. The inhibition of protein synthesis *in vivo* is suggested as a method of demonstrating amino acid antagonism *in vivo* (137).

Small amounts of methionine sulfoximine are deposited in the brain and spinal cord of rats and rabbits; the analogue inhibits the incorporation of methionine in tissues of fed but not fasted rats and rabbits (138). Methionine sulfoximine or D-hydroxylysine inhibit protein synthesis in Ehrlich ascites tumor cells by preventing the synthesis of glutamine (139).

*Biological studies with pyrimidine and purine analogues.*—The inhibitory roles of 5-mercaptopuracil and related pyrimidines and of 5-aminodeoxyuridine are indicated elsewhere (p. 645-6). Inhibitions by 5-fluorouracil in *L. leichmannii* and 5-hydroxydeoxyuridine in *E. coli* are affected by thymidine in a noncompetitive manner over a small range of concentrations, and it was suggested that these compounds inhibit not only the synthesis of thymidine but also other functions of uracil or its derivatives (35, 140). A mutual antagonism of certain pyrimidine analogues such as 5-amino- and 5-hydroxydeoxyuridine in *E. coli* suggests that a common pathway of utilization such as phosphorylation is necessary for these inhibitors to block the utilization of biosynthetic pyrimidines. Depending upon conditions, the deoxyriboside of 5-bromo- or 5-chlorouracil may serve as a growth factor replacing the deoxyriboside requirement or alternatively it may exert an inhibitory effect of its own in the same organism, *L. leichmannii* (141). Azathymine, a competitive antagonist of thymine and thymidine for *S. faecalis* (142), is converted to azathymidine by a transfer of deoxyribose from thymidine (143). Both azathymine and 5-bromouracil strongly potentiate a variety of agents which block the conversion of folic acid to folinic acid. Also, certain combinations of antipurines with antithymines as well as of two antipurines are strongly synergistic growth inhibitors for lactobacilli (144). Azauracil riboside and ribotide accumulate during growth of *S. faecalis* in the presence of 6-azauracil (145, 146, 147); however, no appreciable incorporation into nucleic acids occurs. The toxicity of azauracil is prevented by uracil or uridine, but the analogue riboside is more effective as a growth inhibitor, and is reversed competitively by uridine. Resistance to azauracil is accompanied by inability to synthesize azauracil riboside or to utilize uracil- $C^{14}$  for RNA



synthesis, and resistant strains utilizing uridine are strongly inhibited by the riboside of the analogue. Similar results have been obtained with *L. leichmannii* and sarcoma 180 in tissue culture (148).

6-Mercaptopurine inhibits the incorporation of formate into nucleic acid (149), but it causes an increase in RNA in regenerating rat liver though retarding the regeneration (150). Growth, acetate utilization, formate utilization, and nucleic acid, protein and lipid content, in decreasing order of sensitivity, are all affected by 6-mercaptopurine in *E. coli* (151). 6-Mercaptopurine inhibition of mitosis in sarcoma 180 (152) and lipogenesis in embryo skin fibroblasts (153) is reversed by coenzyme A. Both 6-mercaptopurine and thioguanine inhibit *in vitro* acetylation of sulfanilamide mediated by coenzyme A; the inhibitory effect can be prevented by either ATP or pantothenate (154, 155). DPN synthesis and destruction is inhibited by 6-mercaptopurine, and this effect is partially reversed by adenylic acid (156). Thus, 6-mercaptopurine exerts considerable influence on the biosynthesis and utilization of certain purine-containing coenzymes, if indeed this is not its primary effect. 6-Mercaptopurine-8-C<sup>14</sup> is metabolized by a resistant strain of *S. faecalis* so that the labeled carbon appears in the adenine and guanine of the bacterial nucleic acids (157).

6-Mercaptopurine is converted to its ribotide by interaction with 5-phosphoribosylpyrophosphate in the presence of beef liver inosinic acid phosphorylase (158), and to its deoxyriboside by interaction with deoxyribose-1-phosphate catalyzed by horse liver purine nucleosidase (159). 2,6-Diaminopurine is converted to its riboside by interaction with ribose-1-phosphate catalyzed by liver nucleoside phosphorylase (160). 8-Azaguanine had earlier been reported to undergo this reaction.

Not only is 2,6-diaminopurine converted into the 5'-nucleotide as reported earlier, but it is also metabolized to 2-methylamino-6-aminopurine and its 5'-nucleotide by *E. coli* B. Adenine prevents the formation of these derivatives, and a resistant strain converted the analogue to its methylated derivative but was unable to convert either to 5'-nucleotides (161). While diaminopurine can serve as a source of guanine, it is not incorporated into nucleic acid *per se*. It is oxidized to its 8-hydroxy derivative by milk xanthine oxidase (162) and is a powerful inhibitor of the process of duplication of DNA that precedes mitosis (163).

8-Azaguanine delays the appearance of symptoms of the yellow virus in sugar beets but the plant is also affected (164). The inhibition of xanthine dehydrogenase by 8-azaguanine has been postulated to be significant in the mode of action of this analogue (165).

*Biological studies of vitamin B and coenzyme analogues.*—Rats on a sulfonamide-containing diet excrete a metabolite of histidine, N-formiminoglutamic acid (166), providing the first evidence for the involvement of folic acid in the transfer of a formimino group. The same metabolite appears in the urine of individuals treated with folic acid antagonists (167). The conversion of histidine to glutamic acid in germinating seeds is inhibited by sulfanil-

amide (168). Inhibition studies have led to conclusions that aminopterin prevents the biosynthesis of histidine in *Torula cremoris* (169) and the conversion of deoxyuridine to the thymidine moiety of DNA of chick embryo or bone marrow (170); that sulfonamides inhibit glycine synthesis in *E. coli* (171); and that *p*-aminosalicylic acid inhibits biotin synthesis in *M. tuberculosis* (172).

*p*-Aminosalicylate-inhibited *M. tuberculosis* produces purine intermediates in the medium, analogous to sulfonamide-inhibited *E. coli*, but folic acid and *p*-aminobenzoylglutamic acid reverse the inhibition in contrast to that of *E. coli* (173, 174). *E. stei* incorporates carboxyl-labeled *p*-aminosalicylic acid into a polyglutamate of a modified folinic acid (175), and *p*-aminobenzoate-requiring *E. coli*, grown in the presence of 6-aminonicotinic acid, which substitutes for the growth factor, accumulates a folic acid derivative which may contain aminonicotinic acid in lieu of *p*-aminobenzoic acid (176).

Additional evidence for the inhibition of reduction of the pteridine ring of folic acid by aminopterin comes from the observed accumulation of folic acid and its polyglutamates in place of folinic acid derivatives in *E. stei* inhibited by the analogue (175), and from enzyme studies in which reduction of folic acid by a reduced-TPN dependent enzyme from chick liver was inhibited by the analogue (177). The mechanism of action of a series of 4,6-diamino-1-aryl-1,2-dihydro-s-triazines in some microbiological systems upon the reduction and subsequent utilization of folic acid has been studied (178).

*Aerobacter aerogenes* and *E. coli* inactivate analogues of folic acid with the formation of pteridinecarboxylic acids, pteridinemethanols and *p*-aminobenzoylglutamic acid (179), and *p*-aminobenzoylglutamic acid and 4-hydroxy-2-aminopteridinecarboxaldehyde depress competitively the uptake of folic acid by *E. stei* (180). Cells of *E. coli* grown in the presence of aminopterin and thymine are of normal morphology, but when thymine is omitted, homogeneous populations of long filamentous cells occur (181). Folic acid exerts a protective effect, augmented by vitamin B<sub>12</sub>, on the growth inhibition and inhibition of RNA synthesis by penicillin, in *L. casei* (182).

Both sulfanilamide and  $\gamma$ -(3,4-ureylenecyclohexyl) butyric acid inhibit the utilization of tryptophan by a *N. crassa* mutant requiring nicotinic acid or tryptophan; however, these analogs of *p*-aminobenzoic acid and biotin do not inhibit the utilization of formyl kynurenine. A role of both *p*-aminobenzoic acid and biotin in the conversion of tryptophan to formyl kynurenine has been proposed (183, 184). The effect of certain products of aspartic acid on the inhibition of growth of *L. arabinosus* by biotin sulfone indicate either a role of biotin in their synthesis or a role of the metabolites in biotin utilization (185).

The synthesis of riboflavin by *Eremothecium ashbyii* is inhibited by 4,5,6-triamino and 4,5-diamino-2-hydroxypyrimidine, and the inhibitions are prevented by adenine and xanthine. Azaxanthine also inhibits riboflavin synthesis (186).

Pantethine analogues have some degree of specificity in inhibiting reactions involving coenzyme A. Sulfanilamide acetylation is more sensitive to  $\omega$ -methylpantethine than to *bis* ( $\beta$ -pantoylaminoethyl)disulfide while the reverse is found with citrate synthesis in the same pigeon liver homogenate (187).  $\omega$ -Methylpantothenic acid and oxythiamin inhibitions of ascorbic acid synthesis in germinating *Phaseolus radiatus* are reversed by pantothenic acid (188).

$\omega$ -Methyl analogues of vitamin B<sub>6</sub> perform some functions of the vitamin and inhibit others, as indicated by the ability of the analogues to replace the vitamin B<sub>6</sub> group in promoting growth of *S. faecalis* in the absence of certain amino acids, and to inhibit growth promoted by the vitamin group in the absence of cysteine and L-alanine. The  $\omega$ -methyl coenzyme analogues activate certain transaminases and alanine racemase in cell-free preparations, but have less affinity for the enzymes than the corresponding vitamin B<sub>6</sub> coenzyme; the affinity of the substrate in the tyrosine-glutamic acid transaminase and alanine racemase is diminished by the  $\omega$ -methylcoenzymes (189). The activation of cell-free alanine racemase is inhibited by 4-nitrosalicylaldehyde, 5-deoxypyridoxal, pyridoxal, and pyridoxamine and their corresponding  $\omega$ -methyl analogues, and less effectively by pyridoxine (189). The  $\omega$ -methyl analogues will support growth of rats only for a limited period, after which all of the animals have convulsive seizures, which are infrequent among vitamin B<sub>6</sub>-deficient rats; the dual character of the  $\omega$ -methyl analogues in performing some functions and inhibiting others appears to account for the results observed (190). Deoxypyridoxine inactivates enzymes essential for the incorporation of formaldehyde, or the  $\beta$ -carbon of serine, into methionine in cell-free preparations from sheep liver; pyridoxal phosphate reactivates the system (191).

Of a number of vitamin B<sub>6</sub> analogues, only those containing a 5-hydroxymethyl group and an unsubstituted 6-position were phosphorylated by pyridoxal kinase; however, 2-ethyl-3-amino-4-ethoxymethyl-5-amino-methylpyridine formed a complex with the enzyme but with no evidence of phosphorylation. Some phosphorylated analogues prevent pyridoxal phosphate from combining with tyrosine decarboxylase (192).

Nicotinamidase activity in cell-free extracts of *M. phlei* is strongly inhibited by 3-acetylpyridine, and less so by pyridine-3-sulfonic acid and isonicotinic hydrazide (193). The exchange of nicotinamide of DPN with some nicotinamide analogues, catalyzed by DPNase from pig brain, has been utilized for the synthesis of a number of coenzyme analogues, including the 3-acetylpyridine (194) and nicotinaldehyde analogues. The coenzyme analogues can perform the function of the normal coenzyme in a number of dehydrogenases, but the rates of reaction and equilibrium constants vary, relative to DPN, with different enzymes and the source of the particular dehydrogenase. The acetylpyridine coenzyme reacts with triosephosphate dehydrogenase from two sources, but the nicotinaldehyde coenzyme inhibits the enzymic action (195).

DPN analogues are useful in elucidating the binding sites of dehydrogenases with pyridine coenzymes. For example, an interaction of the reduced acetylpyridine coenzyme analog with liver alcohol dehydrogenase produces a shift in absorption spectrum of the analogue coenzyme similar to that obtained with the coenzyme, and the spectrum of reduced nicotinylaldehyde coenzyme analogue is also altered in the presence of the liver apoenzyme. Amide binding to the apoenzyme is thus excluded from having a role in the spectral shift (196).

The thiamine phosphorylase of yeast has been reported to be inhibited by oxythiamine but not by pyrithiamine; however, the converse has recently been found to be the case with rat liver preparations (197). Inhibition of keto acid oxidases by oxythiamine diphosphate has been extended to  $\alpha$ -keto-butyrate oxidase of wheat germ (198), and triphosphopyrithiamine partially inhibits pyruvic acid oxidase of pigeon breast muscle (199). When administered to pigeons or rats, pyrithiamine decreases the thiamine pyrophosphate content of various tissues while oxythiamine lowers the coenzyme content only in the liver of rats, and in some pigeon tissues (200, 201). Neuromuscular symptoms of avitaminosis were produced only by pyrithiamine. Biosynthesis of a vitamin analogue occurs in *Bacillus aneurinolyticus* which converts 2-ethyl-4-amino-5-aminomethylpyrimidine to the ethyl analogue of thiamine (202).

*Incorporation of analogues into proteins, nucleic acids, and coenzymes.—*

It was recognized some time ago that analogues not only inhibit enzymatic transformations of metabolites, but also may undergo similar reactions. The analogue products have been found to be inert in some cases, to perform some of the functions of the normal products in specific instances, or perform all of the functions of the normal product. Examples of vitamin analogues in all of the categories are known. Since the original observations that purine and pyrimidine analogues such as 5-bromouracil and 8-azaguanine are incorporated into nucleic acid, and ethionine is incorporated into proteins, considerable attention has been devoted to this aspect of metabolite antagonists. The incorporation of an analogue in place of a structural unit repeated in various positions in a large biological molecule could, depending to some extent upon the positions and extent of incorporation, inactivate the molecule, decrease the rate of its performance in a biological function, or form a molecule which acts quite similarly to the original molecule.

Ethionine is incorporated into the proteins not only of rats but also of *T. pyriformis* (203); however, it was not determined whether these proteins have enzymic activities. Abnormal proteins are capable of biological activity in a number of organisms; an interesting example is a mutant of *E. coli* incapable of synthesizing methionine, which in the total absence of methionine grows exponentially and synthesizes enzymatically active proteins in the presence of selenomethionine (204). *p*-Fluorophenylalanine is incorporated into *L. arabinosus* (205), and into *E. coli* (133) with phenylalanine and tyrosine being partially replaced in protein synthesized at a linear rather

than an exponential rate and with inhibition of several adaptive abilities, but not of the synthesis of  $\beta$ -galactosidase. The protein content of a tryptophanless strain of *E. coli* in the presence of 2-azatryptophan (tryptazan), or 7-azatryptophan, doubles before growth stops, and both analogues are found in the protein fractions (206, 207). The mutant does not form a number of adaptive and constitutive enzymes under these conditions, but increased serine deaminase activity and ureidosuccinic acid synthesis is permitted by either analogue. Production of  $T_4$  bacteriophage was inhibited by azatryptophan with infective centers greatly reduced below input values within a short time, and the phage containing the analogue appears to have a defective protein coat.

In Ehrlich ascites tumor cells, thienylalanine, *o*-fluorophenylalanine and ethionine act as competitive substrates of the corresponding amino acids rather than inhibitors of protein synthesis. These amino acid analogues are incorporated into the protein, and do not inhibit the incorporation of amino acids other than their corresponding metabolites into protein (139, 208); *o*-methylthreonine, however, inhibits the incorporation of other amino acids as well as its competitive metabolite, isoleucine, while allo-isoleucine acting as a competitive substrate specifically prevents incorporation of isoleucine. Thus, two modes of action of analogue antagonists can be detected by this methods, one of which involves incorporation of the amino acid analogue into protein.

Incorporation of 5-bromouracil into DNA has been verified by the isolation of its deoxyriboside from DNA of *E. stei* grown in the presence of the analogue (209), and such incorporation occurs in a number of different organisms without appreciable inhibition, or if inhibition occurs, with resumption of growth upon removal of the inhibitor. The replacement of up to 48 per cent of the thymine moieties by 5-bromouracil in DNA of a strain of *E. coli* decreases the viable cells only to 70 per cent of those not treated with bromouracil (210). Although it is possible that all of the DNA is not involved in carrying hereditary functions and only that not involved contains bromouracil, it seems likely that some bromouracil units function just as thymine in the polymeric molecule. Lethal effects of high concentrations of 5-bromouracil are not associated with appreciable incorporation of the analogues into nucleic acid (210, 211). Incorporation of 5-bromouracil to the extent of 79 per cent of the thymine moieties in  $T_2$  coliphage results in 70 per cent of the particles not forming plaques (212).

The size of the 5-halo group in substituted uracils appears to have a role in the degree of incorporation into DNA; 5-iodo- and 5-chlorouracil are incorporated to a lesser extent than 5-bromouracil presumably because the bromo group corresponds most closely to the methyl group of thymine (211, 212). 5-Fluorouracil is incorporated into the RNA but not the DNA of Ehrlich ascites carcinoma, liver, and spleen of mice; however, the biosynthesis of the thymidine moiety of DNA is inhibited by the 5-fluoro analogue of both uracil and orotic acid (213).

6-Azathymine-5-C<sup>14</sup> is incorporated into DNA of *S. faecalis* when the analogue is added either before or during the logarithmic phase of growth. In the former instance, about one-fifth of the thymine of the nucleic acid is replaced by the analogue, while the analogue added during growth is incorporated to a greatly reduced extent with death of all of the cells occurring only under such conditions. Thus, death or inhibition of growth of the organism is not necessarily a result of the incorporation of the pyrimidine analogue into the DNA (214).

8-Azaguanine has been known for some time to be incorporated into a wide variety of organisms. There may be as much as 60 per cent replacement of guanine by the analogue in the RNA of *B. cereus*, but the analogue is incorporated only to a small extent into the DNA of the organism (215, 216). Azaguanic acid has been isolated from hydrolyzed RNA of the organism (217, 218). Addition of 8-azaguanine to proliferating cells reduces the rate of growth within a few minutes to a new growth rate which remains constant after the initial change, and incorporated 8-azaguanine steadily accumulates in the RNA (216). The azaguanine residues are not randomly distributed, since more of them appear in the shorter polynucleotides and at the terminal positions (215). 8-Azaguanosine and 8-azaxanthosine have been identified in ethanol extracts of *B. cereus* (215). 5(4)-Amino-1-H-1,2,3-triazole-4(5)-carboxamide (the aza derivative of aminoimidazolecarboxamide), 8-azahypoxanthine and even 8-aza-adenine are metabolized and incorporated into ribonucleic acid in *E. coli* as 8-azaguanine. Some of these transformations also occur in *B. cereus* and tobacco mosaic virus. The metabolites in general reverse the analogue effects; for example, hypoxanthine can prevent the toxicity and metabolism of 8-azahypoxanthine (215). Tobacco mosaic and turnip yellow viruses show reduced infectivity after replacement of guanine by 8-azaguanine in their RNA (219). 6-Mercaptopurine is measurably incorporated into DNA and RNA of mouse viscera (220).

Thiouracil incorporation to a moderate extent into RNA of tobacco mosaic virus results in reduced infectivity of the virus and can be prevented by supplements of uracil. The reduced infectivity does not result in a decrease in number of infective particles but is due to a decrease in the amount of virus produced from a single particle as a result of a longer lag period in initiating virus production (221, 222). It is proposed that the proportion of elementary units capable of duplication is reduced in each particle; however, difficulties in initiating duplication of abnormal templates may account for a lag phase different from that of the normal templates. Digests of tobacco mosaic virus produced in the presence of thiouracil contain adenylyl- and guanylylthiouridylic acids, thiouridine, thiouridylic acid and a diphosphate of thiouridine (223, 224, 225). Thiouracil is more concentrated at the ends of the polynucleotide chain than in the center, and more so at one end than the other (225).

*L. lactis*, which has been known to grow on lyxoflavin in place of riboflavin to give cells containing lyxoflavin but not riboflavin, contains lyxo-



flavin mononucleotide and dinucleotide (226). The livers of rats fed the riboflavin antagonist, diethylriboflavin, contain diethylriboflavin phosphate (227).

Acetylpyridine in mice is incorporated into a coenzyme analog of DPN, detected by its resistance to the action of DPNase of *Neurospora*. The coenzyme analogue is found in brain, spleen and neoplastic tissues but not in the livers of mice. Acetylpyridine actually causes an increase in the normal coenzyme in liver (228). The coenzyme analogue functions in a number of enzyme systems in place of the natural coenzyme.

An inositolless strain of *N. crassa* inhibited by isomytilitol incorporates the analogue into certain phospholipide fractions in place of inositol (229).

A number of analogues of vitamin B<sub>12</sub> have been prepared by biosynthesis in organisms in which analogues of 5,6-dimethylbenzimidazole or of 1,2-diamino-4,5-dimethylbenzene are incorporated into the vitamin B<sub>12</sub> molecule in place of the dimethylbenzimidazole moiety (230). Vitamin B<sub>12</sub> analogues containing benzimidazole, its 5-methyl, 5-hydroxy, or 5,6-dichloro derivatives and naphthimidazole in place of 5,6-dimethylbenzimidazole have appreciable activity for chicks and are stored in the liver as such without increasing the vitamin B<sub>12</sub> content of liver (231).

*Antagonisms among naturally occurring metabolites.*—Inhibition by a metabolite of its own biosynthesis has been of importance not only in increasing sensitivity of organisms, subcultured in the presence of the metabolite, to analogue inhibition, but also in obtaining sparing effects of end-products on the primary product of an inhibition (82), and is also of importance to isotopic competition methods (232). The mechanisms of this inhibition in general have not been determined. Some involve inhibition of an enzyme essential for its biosynthesis and others involve suppression of the synthesis of such an enzyme. Valine inhibition of its biosynthesis in *E. coli* appears to result from a competitive inhibition of pyruvate utilization for acetolactate synthesis (233). Culturing of *E. coli* in phenylalanine results in increased sensitivity to *threophenylserine* (234).

Phenylalanine inhibits tyrosinase in a melanoma, and such an inhibition may be the cause of diminished pigmentation of phenylketonurics (235). Phenylalanine and phenylpyruvic acid inhibit tyrosine oxidation to acetoacetate in rat liver slices; however, in homogenates, only the keto acid is effective (236). Also, phenylalanine and its keto acid inhibit the incorporation of tyrosine into proteins of rat liver slices (236). Inhibition of phenylalanine and tyrosine synthesis by *p*-hydroxyphenylpyruvic and phenylpyruvic acids, respectively, occurs in *S. faecalis* and *L. arabinosus* (237).

Natural antagonisms in rats have been extended to leucine which exerts an inhibitory effect on utilization of isoleucine and valine, and to antagonisms between isoleucine and valine, phenylalanine and isoleucine, and phenylalanine and valine. Growth retardation by phenylalanine and tyrosine is prevented by threonine at low but not at high levels of the inhibitory amino acids (238). Valine inhibits the synthesis of isoleucine in one strain



of *E. coli*, but in an isoleucine and valine-requiring mutant, antagonism was postulated to occur at the cell surface (239). Growth inhibition by  $\alpha$ -aminobutyric acid and its keto acid is relieved mainly by intermediates of the valine-leucine biosynthetic pathway while  $\alpha,\gamma$ -diaminobutyric acid inhibition is reversed by intermediates involved in methionine and arginine biosynthesis (240).

Lanthionine prevents lysis of a diaminopimelic acid-requiring *E. coli* growing on lysine and suboptimal amounts of diaminopimelic acid, and cystine competitively inhibits utilization of diaminopimelic acid in a mutant of *E. coli* (241). Inhibition by djenkolic acid in *Ochromonas malhemansis* is reversed by homodjenkolic acid which replaces methionine in stimulating growth of the organism (242).

Mutual antagonisms between the vitamin B<sub>6</sub> group and the pyrimidine moiety of thiamine have recently been discovered. 4-Amino-5-hydroxy-(or halo)-methyl-2-methylpyrimidine was previously known to cause convulsions in rats or mice, and this toxicity is prevented by the vitamin B<sub>6</sub> group (243, 244). A similar antagonism occurs in yeast, to affect tryptophan metabolism in rats, and is involved in lethal effects of the pyrimidine derivative in mice (245, 246, 247). The phosphate derivative of the pyrimidine antagonist is a competitive inhibitor of pyridoxal phosphate in tyrosine decarboxylase of *S. faecalis* (248), but does not displace the coenzyme from a number of enzymes known to contain pyridoxal phosphate (249). The opposite antagonism has been shown in the growth inhibition of *Neurospora* by pyridoxine which is competitively reversed by the pyrimidine moiety of thiamin and noncompetitively by thiamine. The evidence suggests that B<sub>6</sub>-like compounds act as competitive substrates (250, 251).

Structural units of reduced TPN, 2'-adenylic acid, 2'-phosphoadenosine-diphosphoribose and even triphosphopyridine nucleotide inhibit certain reactions of the reduced coenzyme, and 2'-adenylic acid inhibits several TPN-linked systems but has no effect on DPN-specific dehydrogenases (252).

*Anomalous antagonisms.*—Structural similarity of an inhibitory compound to a metabolite and reversal by the metabolite is insufficient proof of metabolite antagonism *via* competition for an enzyme site. For example, L-penicillamine ( $\text{HS-C}(\text{CH}_3)_2\text{-CH}(\text{NH}_2)\text{-COOH}$ ) inhibition of rat growth was reversed by ethanolamine and its methylated derivatives. However, inhibition of the utilization of the presumed precursor, serine, for ethanolamine synthesis apparently results not from a competition for the substrate site but from a chemical interaction with the coenzyme of vitamin B<sub>6</sub>. The inhibitor not only causes a pyridoxine deficiency in the intact animals by increasing the excretion of the vitamin, but also interacts with pyridoxal phosphate in enzymes *in vitro*. The chemical interaction of the amino and sulfhydryl groups of the penicillamine with the carbonyl group of pyridoxal phosphate to form a thiazolidine structure is a proposed mechanism for the inhibition (253, 254).

Results with isonicotinoyl hydrazide and vitamin B<sub>6</sub> have the appear-

ance of a competitive antagonism, but the chemical interaction of pyridoxal and its phosphate with the hydrazide was early considered (1). Evidence has now been presented for the direct chemical interaction of the hydrazide with the coenzyme (255), and progressive reaction of isonicotinoyl hydrazide with vitamin B<sub>6</sub> containing enzymes has been observed (256).

Azaserine toxicity for a number of organisms was not reversed competitively by any known metabolite and was accordingly not considered to be a metabolite antagonist. Recently, azaserine and 6-diazo-5-oxo-L-norleucine were found to inhibit the enzymatic conversion of formylglycinamide ribotide to formylglycinamidine ribotide in which glutamine has a role. While azaserine denatures the enzyme, glutamine competitively delays the inactivation suggesting a competitive relationship with a probable chemical interaction of the analogue at the site of complex formation (257).

An interesting metabolite analogue relationship is that of fluoroacetate and acetate. Inhibition of the citric acid cycle by fluoroacetate appears to occur as a result of its conversion to fluorocitrate which inhibits aconitase. Activation of fluoroacetate proceeds with the formation of fluoroacetyl coenzyme A which reacts similarly to acetyl coenzyme A in some of its enzyme systems including the citric acid synthesizing enzyme (258, 259). However, acetate-activating systems do not seem to activate fluoroacetate; so it appears that in systems such as guinea pig kidney particles, in which acetate is capable of preventing the formation of fluorocitrate from fluoroacetate, the metabolite and its analogue are metabolized separately to intermediates, presumably coenzyme A derivatives, which have a competitive relationship rather than such a relationship occurring with the simpler substances (260).

*Antimetabolites in the study of development and differentiation.*—Antimetabolites have found a rather prominent position in recent investigations seeking biochemical explanations for the biological changes which occur during development and differentiation. Their use is based on the assumption that blocking the utilization of a specific metabolite will lead to the failure of developmental processes which are directly dependent upon the particular metabolite. This assumption is not without criticism, since anything which interferes with the over-all metabolic activity of an organism will slow growth and development along with everything else. However, in cases where it can be shown that a particular phase of development is retarded disproportionately to the effect on the organism as a whole, it is not unreasonable to postulate a special role of the corresponding metabolite in the developmental process.

Developmental systems usually will not tolerate the large ranges of concentration necessary to prove a competitive inhibitor-metabolite relationship. However, in view of the many nonspecific developmental effects caused by detergents, dyes, anaesthetics, hormones, sulfhydryl reagents, chelating agents, etc., there is no justification for assuming the effects of any compound are due to metabolite antagonism until reversal with at least one concentration of a natural metabolite is demonstrated.

Antimetabolites have been applied to most of the test systems used in the study of development and differentiation. A few of the most interesting studies will be discussed.

Regeneration of *Hydra* is reversibly inhibited by a number of antimetabolites including  $\beta$ -2-thienylalanine, 5-bromouracil and 4-aminopyrazolo (3,4-d)pyrimidine, without apparent serious side effects on the organisms. Application of the analogues for four-hour intervals at various times during the eighteen-hour regeneration period revealed that the analogues are effective only during specific intervals which are characteristic of the type of inhibitor used. These data have been combined with time-of-effect data for various other physiologically active compounds and with cytological observations to yield a tentative time sequence for a number of the major events in the regeneration of hydra, including the participation of protein and nucleic acid metabolism (261).

Although preliminary, this system illustrates well the potential value of antimetabolites in correlating biochemical changes with physical and physiological changes during development. Similar studies are theoretically possible in any system where an antimetabolite can be applied for a short time and then be effectively removed (or fully reversed with a natural metabolite). The degree of correlation with specific physiological or cytological changes which can be accomplished will decrease sharply, however, with the increasing complexity of the biological system, since the number of developmental changes occurring simultaneously rapidly increases, and it becomes more and more difficult to correlate any one of them specifically with the biochemical observations. Structural analogues of vitamins, amino acids, and nucleic acid derivatives cause animalization, vegetalization and radialization of developing sea urchin embryos (262, 263). Unfortunately, reversals with natural metabolites were not reported. However, if antimetabolite action can be proven, the very potent animalizing action of 2-thio-5-methylcytosine will be of great interest.

Four different phases of development in *Rana pipens* embryos are particularly sensitive to antimetabolites. Various compounds stop development at Shumway stages 8-9 (blastula), 11-12 (gastrula), 14-15 (neurula), or 20-23 (hatching to opercular fold formation), but not at intermediate stages. Bacterial purine antagonists stop development at early stages, while antagonists of folic acid or citrovorum factor tend to inhibit at neuralation. Unfortunately, this preliminary report which mentions testing of over 100 compounds presents very little specific data for individual compounds and almost no data on reversals (264).

It was also noted that among the embryos treated with analogues of nucleic acid derivatives there were cases where epithelial papillae instead of a normal lens were induced when the optic vesicle came into contact with the overlying ectoderm. This is very interesting in view of the postulated role of nucleic acids in induction (265). If it can be shown that the nucleic acid content of the inducing tissue was indeed altered, antimetabolites may become powerful tools in studies of inducing substances. Antimetabolites

were used extensively in demonstrating the role of phenylalanine in the differentiation *in vitro* of the cranial neural crest region of *Ambystoma maculatum* embryos (266). Alteration of the phenyl group blocks differentiation of the enteromesenchyme, and replacement of the  $\alpha$ -amino group with an  $\alpha$ -hydroxy group interferes with pigment cell differentiation. Exogenous phenylalanine, or phenylalanine synthesized by the archenteron roof mesoderm from exogenous precursors will reverse the effects. From these studies, it was concluded that phenylalanine provided by the archenteron roof mesoderm is essential for the normal differentiation of the neural crest, and that this may represent at least a partial controlling mechanism for this differentiation process.

The effects of a series of leucine analogues on the chick embryo both *in situ* and *in vitro* have been carefully compared with those of a methionine analogue. Each causes a characteristic developmental pattern, suggesting different requirements for amino acids by different development processes (267). These studies suggest that differential supplies of simple metabolites may be very important in the regulation of developmental processes.

It was known long before the antimetabolite concept was developed that maternal nutritional deficiencies could have teratogenic effects on both avian and mammalian embryos. A large number of antimetabolites have been used to duplicate these effects; the major points of this work have been reviewed (268, 269). The general effect of antimetabolites in these systems is fetal death at high concentrations with stunting and teratogenesis at lower concentrations. In rats, a number of antimetabolites cause fetal resorption after implantation but do not block implantation (270). Aminopterin caused no teratogenesis in the surviving embryos.

Use of the embryo-destroying properties of antimetabolites for therapeutic abortion in humans has been suggested, particularly in cases where it is desirable to avoid the use of anaesthesia. Preliminary tests with aminopterin and azaserine show promising results, although the compounds were not 100 per cent effective and caused some severe side reactions in highly sensitive subjects (271).

Antimetabolites have already proven themselves to be of great value in the study of factors controlling development, particularly in such notable experiments as the blocked lens induction and the demonstration of the role of phenylalanine in neural crest differentiation. How far such studies will go toward elucidation of the ultimate controlling mechanisms of development seems at this time to be limited by only one factor—the degree of importance of simple metabolites and their metabolic products in the controlling mechanisms.

*Antimetabolites in tumor and other chemotherapy.*<sup>4</sup>—The success of antimetabolites in chemotherapy has been considerably less than anticipated just

<sup>4</sup> Interesting pharmacological studies of effects which may have an antimetabolite basis have been omitted, e.g., studies of antiserotonins in hypertension [D. W. Woolley and E. N. Shaw, *Science*, **124**, 34 (1956)].

after the discovery of the mode of action of sulfonamides; however, some progress has been made, particularly in the field of chemotherapy of tumors, the subject of a number of monographs (272, 273) and reviews covering such aspects as employment of methods of inhibition analysis in the normal and tumor-bearing mammalian organism (274), folic acid and purine antagonists in chemotherapy (275, 276), the development of resistance to these agents (277), and their effects on nucleic acid metabolism (278).

Increasing interest in amino acid analogues has resulted from the discovery of two antibiotics, azaserine and 6-diazo-5-oxo-L-norleucine, which inhibit growth of mouse sarcoma 180 (279, 280). Resistance to azaserine inhibition of glycine incorporation into purines, observed in intact cells of a strain of a mouse neoplasm, is not found in cell-free extracts converting formylglycinamideribotide to aminoimidazoleribotide (281). Ethionine inhibits a rat sarcoma (282) and enhances the effects of a folic acid antagonist on a mouse mammary carcinoma (283). Depression by 3-thiophenealanine of the growth of sarcoma transplants and of antibody formation in rats is reversed by phenylalanine (284). The ability of amino acid analogues to inhibit virus reproduction has been extended to *L-threo*-phenyl-serine inhibition of influenza virus growth in tissue culture (285), and to  $\beta$ -2-thienylalanine, ethionine and 6-methyltryptophan inhibition of psittacosis virus growth without toxicity for the chick embryo host cells (286).

Among pyrimidine analogues, certain fluoro derivatives such as 5-fluorouracil and 5-fluoroorotic acid have recently been found to be markedly inhibitory to development of a variety of rat and mouse tumors (287). 6-Aza-uracil riboside inhibits sarcoma 180 growth (153, 156), and azathymidine decreases the incorporation of formate into DNA thymine in bone marrow or ascites tumor cells *in vitro* (288). 5-Aminouridine reduces phosphate incorporation into rat liver and hepatoma (289). The inhibitory effects of a series of pyrimidine analogues upon poliomyelitis virus in monkey tissue culture have been reported (290).

Among new purine analogues, 2-fluoroadenosine appears promising, since it inhibits human epidermoid carcinoma (33). 4-Aminopyrazolo(3,4-d)pyrimidine and its 1-methyl derivative inhibit growth of a mouse adenocarcinoma and also increase the life span of mice with certain leukemias (49). Many of the well-known purine analogues are of benefit in treatment of acute and chronic myelocytic leukemias, particularly 6-mercaptopurine (276, 273), thioguanine, which shows no advantages over 6-mercaptopurine (276), and 6-chloropurine, which, however, appears to share cross-resistance with 6-mercaptopurine (276, 291); these analogues have been found effective against a number of experimental tumors. In contrast to earlier results, resistance to 8-azaguanine in leukemia cells of mice appears to be not necessarily correlated with azaguanine-deaminase activity (292); the potentiation of azaguanine by aminoimidazolecarboxamide appears to result from the inhibition by the imidazole of guanase, which destroys azaguanine (293). The riboside of 6-mercaptopurine (294) and ribotide of 8-azaguanine (295) show

no increased antitumor activities over those of the parent compounds. As determined by phosphate incorporation, 8-azaguanine and 6-mercaptopurine depress DNA synthesis in a mouse adenocarcinoma (296). Unsubstituted purine is more toxic to mouse sarcoma than to mouse skin in tissue culture (297); it retards growth of sarcoma 180 in mice (291).

Suitable administration of folic acid antagonists produces beneficial effects in acute leukemia of children with remission lasting from two weeks to more than six years, with a median of eight months (276). Besides toxic effects, the principal drawback of such therapy is resistance development; cross-resistance is usual among the various folic acid antagonists, but antifolic acid resistance is not necessarily concomitant with resistance to other antitumor agents (276, 273, 298). In leukemia cells, resistance to amethopterin is associated with diminished ability of the analog to inhibit nucleic acid synthesis (299) or to depress formate incorporation into protein (300) and purines (301); chronic granulocytic human leukemia cells are more sensitive to amethopterin inhibition of formate incorporation than acute leukemic cells, while little or no inhibition occurs with chronic lymphocytic or normal leucocytes (302). Of considerable interest is the ability of DNA extracted from strains of *Diplococcus pneumoniae* resistant to amethopterin to induce resistance in susceptible strains (303). The reported inhibition by aminopterin of propagation of influenza virus in tissue culture (304) suggests the question whether this is the result of a detrimental effect on the tissue which in turn impedes virus reproduction, rather than a differential toxicity for host cells and virus; on the other hand, a toxicity differential appears to exist in combination therapy with sulfadiazine and pyrimethamine for toxoplasmosis, in which the hematologic toxicity is prevented by folic acid, which, however, is not utilized by the *Toxoplasma* as a reversing agent for the inhibitors (305). 4-Aminopteroyl-amino adipic acid arrests growth of a leukocytoma transplant (306).

Analogues of vitamins other than folic acid have on occasion been found to show antineoplastic effects; early reports included certain riboflavin and vitamin B<sub>6</sub> analogues. The tumor-promoting effects in *Drosophila* of particular vitamins are inhibited by their corresponding analogues (307). 6-Aminonicotinamide inhibits a lymphosarcoma and certain adenocarcinomas (308, 309). 5,6-Dimethylbenzimidazole and related compounds inhibit growth of *E. histolytica*, and vitamin B<sub>12</sub> or diaminodimethylbenzene is reported to prevent the inhibition (310).

Chemotherapy by combinations of vitamin analogues or other antimetabolites has received increasing laboratory study. The employment of such combinations of drugs has recently been reviewed (311).



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## BIOCHEMISTRY IN THE U.S.S.R.<sup>1</sup>

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By publishing a large number of reviews and surveys of the major advances which have been made, mainly abroad, in the fields of chemistry, biochemistry, microbiology, medicine, tracer methodology, etc., the Soviet Union demonstrates the availability to the Soviet scientists of foreign literature in the original and in translated form. The rapid appearance of foreign books and annual survey publications, mainly from the United States, efficiently and accurately translated into Russian, also indicates the operation of an efficient organization in which properly qualified personnel is engaged in a very useful activity. The availability to the Russian scientists of basic findings which are essential for practical development and application certainly spares the Soviet Union considerable manpower and expenditure, and releases the corresponding number of qualified personnel for practical and more immediate activities. To our mind, here lies the secret of rapid and "spectacular" advances in practical developments, which are and always were more obvious and more publicized than spadework of a basic nature. Translations from Russian into English, including these reviews, are an attempt to acquaint the research worker outside of the Soviet Union with the advances that are being made by biochemists who reside in the Soviet orbit.

As in the previous review, not all the published material could be included here. Only that which appeared to the reviewer as the most significant was considered, as carefully and as thoroughly as the exigencies of space permitted.

**Proteins.**—Gorbacheva *et al.* (1) have shown that the character of morphological changes of the macromolecular structure of horse serum albumin and of  $\alpha$ -chymotrypsin during denaturation depend on the structure of the protein and on the specificity of the denaturing agents and stabilizers. The macromolecules of serum albumin retain their capacity for reversible denaturation while their internal S—S bonds remain intact. Disruption of these internal S—S bonds, with accompanying transulfuration, results in irreversible denaturation and aggregation. In the presence of stabilizers the disruption of the S—S bonds is not accompanied by aggregation, and yet the resulting morphological changes are irreversible. At extreme pH values the protein behaves as a linear polyelectrolyte. Denaturation of  $\alpha$ -chymotrypsin proceeds abruptly. Lack of a disulfide framework facilitates denaturation and unless the configurational changes are accompanied by the disruption of chemical bonds, restoration of the original structure can be effected upon removal of the denaturing agent.

<sup>1</sup> The survey of the literature pertaining to this review was completed in October, 1957.

Chernikov (2) reported that the stability of native serum albumin towards the action of an individual proteinase is greatly reduced in a mixture of trypsin, chymotrypsin, and carboxypeptidase. The initial rate of hydrolysis is a function of the ratio of the enzyme concentration to substrate concentration. The maximum rate of hydrolysis was observed when a 1 per cent solution of protein was subjected to the action of a mixture of trypsin (enzyme to protein, 1:25), chymotrypsin (enzyme to protein, 1:100), and carboxypeptidase (enzyme to protein, 1:10). Trypsin split about 224 peptide bonds of albumin; chymotrypsin, about 156; and a mixture of the two, about 235. These figures are independent of hydrolysis time. When trypsin and carboxypeptidase, or chymotrypsin together with carboxypeptidase, or all three enzymes together were allowed to act, the number of peptide bonds split was proportional to the time of hydrolysis. It was concluded that enzymatic hydrolysis of a native protein consists of two qualitatively different stages: a slow proteolytic stage, and a faster peptolysis stage. Burnett & Kennedy (3) demonstrated the existence of a soluble enzyme system in liver which brings about the phosphorylation of casein in the presence of a high energy phosphate donor. Sundararajan *et al.* (4) now report a similar system existing in the mammary gland of the lactating rat which brings about the phosphorylation of casein or of dephosphorylated casein in the presence of ATP. Alternate paths for the biosynthesis of phosphocasein have been explored, and no evidence was obtained to show a direct phosphorylation of serine even though traces of phosphopeptides were found in the lactating mammary gland. Phosphoserine, however, can arise from glucose (5), and the phosphopeptides could serve as the primary intermediates in the biosynthesis of casein. This is an alternate pathway of casein synthesis which contrasts with the direct phosphorylation path observed in these experiments.

Troitskii & Tarasova (6) examined the blood serum proteins of atherosclerotic rabbits (cholesterol fed) by three methods: electrophoresis in the optical instrument developed by Troitskii (7) with veronal buffer of pH 8.6 and ionic strength of 0.1 (8); paper electrophoresis, using the same buffer but with ionic strength of 0.05; and salt fractionation (9). Cholesterol was determined in the beginning and at the end of the experiments by the method of Troitskii & Tarasova (9). With development of atherosclerosis, the method of salt fractionation showed no difference from the normal blood picture. Optical electrophoresis showed an increase in  $\beta$ -globulins and a decrease in albumin. Paper electrophoresis showed a decrease in albumin and an increase in  $\alpha$ - and  $\beta$ -globulins in atherosclerotic rabbits. Ether extraction of blood serum prior to analysis revealed that by all three methods there are no changes in the protein content of any blood fraction of atherosclerotic rabbits. The highest cholesterol content in blood from atherosclerotic rabbits was associated with the  $\beta$ -globulins, which have been shown previously by Troitskii (10) to possess a high absorbing capacity for large hydrophobic colloidal particles of lipides. Citral and vikasol, administered together with cholesterol, markedly inhibit atherosclerotic development in rabbits and

lower the hypercholesteremia. This is explained by the fact that citral acts directly on  $\beta$ -globulin and thus prevents the cholesterol from associating with  $\beta$ -globulin. Lecithin was without effect on atherosclerosis development.

Troitskii & Sorkina (11) reported that formaldehyde, acetaldehyde, or lactaldehyde, administered intravenously or subcutaneously to rabbits, induce an increase in the  $\alpha$ - and  $\beta$ -globulins of blood serum. Additional globulins appear in the  $\alpha$ -globulin region. The same results were obtained on incubation of blood serum with the aldehydes. The appearance of additional fractions in the  $\alpha$ -globulin region was established immunologically. Benzoquinone and quinhydrone also induced similar changes in the composition of blood serum. The authors suggest that as a result of the high adsorption properties of  $\alpha$ - and  $\beta$ -globulins, adsorption complexes arise between the altered proteins and the basic serum proteins, and that the altered electrophoretic pattern is a reflection of the formation of these complexes. The alteration of serum proteins by the aldehydes could be chemical (interaction of active protein groups with aldehydes) or a result of denaturation. Tarasova (12) examined blood serum proteins of normal and atherosclerotic humans by salt fractionation and electrophoresis. Salt fractionation showed no quantitative differences in the content of albumin,  $\alpha$ - and  $\beta$ -globulins in these individuals. Electrophoretic analysis, on the other hand, showed a decrease in albumins and an increase in the  $\alpha$ - and  $\beta$ -globulins in atherosclerotic humans. Simultaneous analysis of these fractions for cholesterol revealed that the cholesterol bound to the  $\alpha$ - and  $\beta$ -globulins was not altered in atherosclerotic patients while that bound to albumin increased by about 100 per cent.

Sumtsov (13) fractionated human milk proteins by paper electrophoresis. The best results were obtained with Leningrad chromatographic paper M-2 in medinal-veronal buffer, pH 8.4, ionic strength 0.17, at 175 v. and 0.2 m.amp./cm. for 25 hr. Developers were bromphenol blue, 0.5 gm.;  $\text{PbCl}_2$ , 10 gm.; cold acetic acid, 20 ml., and water up to 1 l. The procedure permitted separation of  $\alpha$ -casein,  $\beta$ -lactoglobulin and immunologically active globulins;  $\gamma$ -casein and lipoproteins remained at the origin, and  $\beta$ -casein moved along with the immunologically active globulins. Lipase activity moved towards the anode, near the  $\gamma$ -globulins. Lipase is found in the water phase and is either a globulin or is associated with immunologically active globulins. Galaev & Alimova (14), employing paper chromatography followed by ionophoresis, determined the amino acid composition of lipoproteins of white and grey human brain. The following amino acids were found in lipoproteins of both tissues: cystine, arginine, lysine, histidine, aspartic and glutamic acids, threonine, serine, alanine, tyrosine, phenylalanine, and valine. Employing a butanol-acetic-water (4:1:5) system, another unidentified amino acid was located just ahead of serine. Novikova *et al.* (15), employing a specially designed apparatus which is described in the paper, separated the intracellular particles by precipitation through liquid layers of varying density. The degree of purity of the four isolated fractions of particles was ascertained in the electron microscope and by examination for RNA and succinodehy-

drase activity. Fraction 1 was free of RNA and succinodehydrazase activity; it was water-soluble and contained iron. Fraction 2 were mitochondria containing maximal amounts of succinodehydrazase activity, and with membranes lysable by water and containing RNA. Fraction 3 were glycogen, and fraction 4 was microsomes containing, in high amounts, RNA which, in contrast to the RNA found in fraction 2, was not lowered by starvation.

Kretovich *et al.* (16) studied lyophilized preparations of albumin and globulin of soya bean in the ultracentrifuge and found that the albumin fraction consisted of a homogeneous protein with an admixture of polydispersed protein of relatively low molecular weight. The sedimentation constant of the former was  $1.98 \pm 0.003$  Svedberg units. The molecular weight of the basic component was 16,000; of the polydispersed admixture, 5,000. The globulin fraction consisted of two independently sedimenting components with sedimentation coefficients of 14.0 and 8.0 S, respectively. The molecular weight of the components was 330,000 and 126,000, respectively. In the presence of cysteine, component  $S_8$  gave a dimer with  $S=10.7$  and mol. wt. = 245,000. The appearance of the dimer was associated with the formation of unknown labile bonds, since the addition of NaCl to the dimer restored it to the original state.

Orehovich & Shpikiter (17) determined various physical constants for pepsinogen and pepsin which were prepared by the methods of Herriott (18) and Northrop *et al.* (19). The sedimentation constant for pepsinogen was  $S=3.60 \times 10^{-13}$  sec. and for pepsin  $S=3.25 \times 10^{-13}$  sec. The diffusion constant for pepsinogen was  $D=7.54 \times 10^{-7}$  cm.<sup>2</sup>/sec. and for pepsin,  $D=8.70 \times 10^{-7}$  cm.<sup>2</sup>/sec. The latter values permitted an evaluation of homogeneity by the method of Dieu & Oth (20). The partial specific volume for pepsinogen was 0.726 cm.<sup>3</sup>/gm. and for pepsin, 0.725 cm.<sup>3</sup>/gm. The molecular weights, calculated by Svedberg's formula were, for pepsinogen 42,240 and for pepsin 32,930. The degree of asymmetry of pepsinogen was greater than that of pepsin; this suggests that in the process of activation, i.e. transformation of pepsinogen to pepsin, a portion with a molecular weight of 9000 is cleaved from the molecule. Orehovich *et al.* (21) determined the terminal amino acids in pepsinogen [N-terminal by the method of Sanger (22)]. In agreement with Herriott (23), the molecule of pepsinogen was postulated to be a single polypeptide chain with N-terminal leucine and C-terminal alanine. Pepsinogen, however, proved to be inhomogeneous on free electrophoresis. It consisted of several components, which, after transformation, gave rise to pepsins of equal activity.

Nagai *et al.* (24) found that actin inhibited myosin ATPase activity in concentrations of KCl higher than 0.2 M and that this inhibition may be attributed to the  $Mg^{++}$  present in actin. In concentrations of KCl lower than 0.2 M, actin activated myosin ATPase activity and this activation may be attributed to actin itself. The results further showed that at high concentrations of KCl, the addition of ATP results in the physical dissociation of acto-myosin into actin and myosin. G-actin did not affect the activity of myosin

ATPase at low concentrations of KCl, and polymerized actin activated myosin ATPase, the extent of activation being a function of the degree of polymerization of actin. The activation of ATPase activity of H-meromyosin and acto-H-meromyosin by  $\text{Ca}^{++}$  increased with a decrease in the concentration of KCl.  $\text{Mg}^{++}$  inhibited the activity of H-meromyosin and of acto-H-meromyosin ATPase. It is suggested that the principal ATPase which participates in muscular contraction is actomyosin-ATPase and not myosin-ATPase.

Salganic (25) finds that the extent of incorporation of methionine- $\text{S}^{35}$  into the blood serum proteins of rats *in vitro* is a function of the age of the animals; rats, 1.5 months of age, incorporated nearly 40 per cent more  $\text{S}^{35}$  than to animals 20 to 24 months old. Sisakiyan & Gumilevskaya (26) report that during metamorphosis of *Bombyx mori* of Ukrainian strain No. 1 there occurs an intense mineralization of acid-soluble phosphorus-containing compounds during histolysis and histogenesis. During the metamorphosis of the intact pupa there is an accumulation of nucleic acids accompanied by an increase in DNA and a decrease in RNA, and an accumulation of purine base nitrogen. A marked decrease in protein content is evident toward the end of development.

Sisakiyan & Filipovich (27) studied protein synthesis in the isolated structures of plant cells; a factor which inhibits the incorporation of glycine-1- $\text{C}^{14}$  into chloroplast proteins was detected in the centrifugate obtained on fractionation of a homogenate of green leaves. This factor, however, was ineffective in protein synthesis in animal cells. Although the incorporation of radioglycine into chloroplast protein was not stimulated by acids of the Krebs cycle, it was inhibited by ATP. In this respect protein synthesis in plants differs from that in animal cells. Protein synthesis occurs in all types of protoplasmic structures of the plant cell, the rate of synthesis varying from structure to structure. The maximal incorporation of radioglycine occurs in mitochondria. Only the chloroplast fraction, however, is capable of a net increase in protein production.

Zbarsky & Perevoschikova (28) found that there is no marked difference in the extent of incorporation of methionine- $\text{S}^{35}$ , glycine-1- $\text{C}^{14}$ , or tyrosine-1- $\text{C}^{14}$  into the nuclei and whole tissue proteins of rat and mouse liver, spleen, and kidney, and of the rat thymus. By contrast, the cell nuclei of transplanted tumors (rat sarcoma  $\text{M}_1$ , mouse Ehrlich-ascites carcinoma, and mouse C3H A hepatoma) showed a much lower extent of incorporation of the same amino acids than the whole tissue proteins of the tumors. In normal cell nuclei the highest rate of incorporation was found in the "acid protein" fraction, the nucleoprotein and residual protein fractions being less active in this respect. In tumor nuclei the "acid protein" fraction was less active than the nucleoprotein fraction, while the residual protein fraction showed little activity. It is proposed that cell nuclei and cytoplasmic particles play different roles in protein synthesis, the nuclei synthesizing more of the specific proteins needed for normal development and differentiation. The im-



pairment of amino acid incorporation by the tumor nuclei proteins is considered evidence of nuclear damage and an alteration in protein synthesis, which are characteristic of dedifferentiation and anaplasia.

Spasskaya (29) reported that methionine- $S^{35}$  inhibits the development of transplanted rat sarcoma 45 by 45 to 67 per cent, and of rat sarcoma M<sub>1</sub> by 33 to 77 per cent. The best therapeutic effects were obtained with doses of 400  $\mu$ c. during the first 10 days. The dose could then be reduced to 200  $\mu$ c. applied every other day. Methionine- $S^{35}$  produced similar effects on Brown-Pearce sarcoma in rabbits, but it was ineffective against mouse lymphoma. No changes in bone marrow were noted. Petrov & Nosova (30) report that while *B. coli* and *B. Breslau* cannot synthesize methionine in the absence of vitamin B<sub>12</sub>, they can grow in a methionine-containing medium free of vitamin B<sub>12</sub>. High radioactivity was found in bacteria cells grown with methionine- $S^{35}$ . The activity was lowered if vitamin B<sub>12</sub> were added to the medium containing radiomethionine since the ability of the bacteria to synthesize nonradioactive methionine was restored. Even when growing conditions were identical and the B<sub>12</sub> content was as high as 1  $\mu$ g. per ml. of medium, the radioactivity of the deficient bacteria was more than twice as high as that of those bacteria which were capable of synthesizing methionine in the absence of B<sub>12</sub>. Attention is called to the fact that in order to lower by addition of vitamin B<sub>12</sub>, the radioactivity of deficient bacteria grown on radiomethionine, it is necessary to add thousands of times more B<sub>12</sub> than is present in a medium minimal in respect to B<sub>12</sub> yet permitting methionine synthesis and maximal growth.

Pawelkiewicz & Nowakowska (31) obtained six new compounds synthesized by *Propionibacterium shermanii* and having the following bases present in nucleotides: benzimidazole, 5-nitrobenzimidazole, 5(6)-imidazo-benzimidazole, 5(6)-nitro-6(5)-methylbenzimidazole, 5(6)-dinitrobenzimidazole, and 5-ethoxybenzimidazole. The compounds were active as B<sub>12</sub> sources for *Escherichia coli* and *Euglena gracilis*. Skarzynski *et al.* (32) reported that paper electrophoretic and microbiological studies revealed an appreciable portion of serum cobalamine to be combined with proteins, mostly with the  $\alpha$ -globulin fraction. "Erythroglubulin" was proposed as the name for the fraction which binds the cobalamine. In the erythrocytes the major portion of cobalamine is bound to insoluble proteins, and in the liver and kidney of the rat cobalamine is found in mitochondria almost entirely in the form bound to proteins. Ostrowski & Niewiarowska (33) further purified the "erythroglubulin" by dialysis against water, fractional precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and paper electrophoresis, obtaining a 625-times concentrated product which proved to be homogeneous. As before (32), erythroglubulin was found in the  $\alpha$ -globulin fraction of bovine blood serum.

Lodin & Kolousek (34) noted that 24 hours after injection the uptake of methionine- $S^{35}$  was greater in the gray matter than in the white matter of the spinal cord, cerebral cortex, and basal ganglia. In the cerebellum greater activity was noted in phylogenetically old regions than in young regions.

The motor neurons of the anterior spinal horns had a higher radioactivity than the remaining gray matter of the spinal cord. Administration of methionine sulfoximine, four hours after injection of radiomethionine, inhibited the uptake of the latter, the greatest inhibition being in the cerebellum.

Orekhovich & Pavlikhina (35), employing glycine-1-C<sup>14</sup>, studied at intervals ranging from 3 to 960 hr. the synthesis of collagen from procollagen in normal and scorbutic guinea pigs. In normal guinea pigs the activity of procollagen decreased with time and that of collagen increased. In deficient animals the extent of incorporation of glycine-1-C<sup>14</sup> activity was inhibited and with time, little if any, activity in collagen was observed. The incorporation of radioactivity into proteins of other tissues, however, was not inhibited by deficiency in ascorbic acid. Administration of ascorbic acid restored procollagen and collagen synthesis. These conclusions confirm the observations of Robertson & Schwarz (36) who found that the collagen of skin decreases in scorbutic guinea pigs, and that ascorbic acid plays a vital role in the synthesis of proteins of the skin.

Sorm *et al.* (37), on the basis of studies of 68 known protein structures or their fractions, found some regularities (notably a certain selectivity of the bonds between the amino acids, the occurrence of identical peptide sequences in different proteins, and the repetition of identical or structurally related amino acids in peptide chains) which suggest the existence of a certain order in the geometrical arrangement of the peptide chains. A comprehensive tabular survey of the types of bonds so far described is expressed in the form of di- and tripeptide sequences. Another table gives the relative frequency of peptide bonds in various proteins. Closer studies are presented of proteins that are functionally identical but of different origin and *vice versa*. The authors suggest that proteins produced by a particular organ, and which fulfill similar hormonal, enzymic, or other function in the organism are probably synthesized in the same way by a definite type of protein pattern peculiar to the organ.

Aliverdiev (38) reports that rats during fetal development do not produce antibodies in spite of the injection of antigens. Chicken embryos also fail to develop antibodies from injected antigens. The same is true for newborn goats. It would appear that the immunological activity of offspring is an essential condition for the survival of the young in a medium which often contains substances which could act for the embryo as antigens. For these reasons the vaccination of newborn has no practical value.

*Carbohydrates.*—Sakshevskii *et al.* (39) report that homogeneous fractions of dextran in borate solutions at pH 10 show an electrophoretic mobility which is proportional to the molecular weight of dextran in the range of 18,000 to 60,000. This mobility of dextran depends on the molarity of the borate; the greatest difference in the mobility of various fractions of dextran was observed in 0.045 to 0.085 *M* buffers. In lower or in higher concentrations the differences in mobility were less pronounced. It was possible to separate dextrans of different molecular weights by electrophoretic partition.

The number of borate residues per glucose residue was considerably greater than 1.5, and this excludes the formation of cyclic borate-diol compounds. The data are in conformity with the hypothesis that each hydroxyl of glucose is bound with one borate residue. Petrova (40) incubated, aerobically, slices of liver with glucose ( $2 \times 10^{-4}$  M) in a salt buffer containing  $K^+$ ,  $Mg^{++}$ ,  $Ca^{++}$ , and dinitrophenol. The presence of DNP did not inhibit glycogen formation which was of the order of 3 mg. per hour per gram of liver. The concentration of ATP was decreased in the presence of DNP. Extracts of liver, although practically free of phosphorylase and phosphoglucomutase activity, also synthesized glycogen (1 mg. per hr. per ml. of extract). Heating of the extracts at  $100^\circ$  abolished the synthesis of glycogen. The formation of glycogen by the extracts was not accompanied by changes in the amounts of hexosemonophosphate esters therein. Petrova concludes that the synthesis of glycogen by liver slices or liver extracts does not proceed exclusively via the hexokinase  $\rightarrow$  phosphoglucomutase  $\rightarrow$  phosphorylase pathway.

Rosenfeld *et al.* (41) and Rosenfeld & Lukomsкая (42) have previously shown that various animal tissues contain an enzyme, named 1,6-glucosidase, which cleaves the 1,6-bonds of dextran to yield glucose. The wide occurrence of this enzyme in mammalian tissues suggested the occurrence of a polysaccharide of dextran type in animal tissues. From "aqueous" and "alkaline" extracts of liver, treated with amylase to cleave glycogen, Rosenfeld & Lukomsкая (43) removed the proteins, and dialyzed the filtrate for 24 to 48 hr. The alcohol precipitable material was purified by recrystallization from aqueous ethanol and dried. The iodine reaction on the material was negative; the anthrone reaction was positive. Hydrolysis in 5 per cent HCl or  $H_2SO_4$  yielded 96 to 100 per cent glucose, which was identified chromatographically and by fermentation with yeast. In 0.5 N HCl (incomplete hydrolysis) isomaltose predominated, in contrast to maltose obtained from glycogen under similar conditions. Apparently, in this preparation there were more 1,6-glucosidic linkages than in glycogen. The preparation, when subjected to the action of dextran-1,6-glucosidase obtained from ox spleen, formed glucose (78 to 93 per cent in 48 hr.). In 1 hr.  $\alpha$ -amylase cleaved only about 10 per cent of the material to glucose as compared to 60 per cent of glycogen. It is possible that the product was  $\alpha$ -dextrin, formed by the action of amylase on liver glycogen. It is known, however, that amylolysis of branched polysaccharides leads to the formation of polysaccharides. The latter are removable by dialysis and have a coefficient of polymerization not exceeding 8 to 10 (44, 45). Khaikina (46) injected glucose- $U-C^{14}$  subcutaneously into guinea pigs and sacrificed the animals 90 min. later.<sup>3</sup> Free glycogen, glycogen bound to lipides, and glycogen bound to proteins were isolated from the brain. Lipide-bound glycogen amounted to 16 mg. per 100 gm.; total glycogen, 70 mg. per 100 gm.; free glycogen, 13 mg. per 100 gm. The greatest specific activity was found in free glycogen, followed by that bound to lipides, then by that bound to proteins. The differences in the rates of synthesis of these glycogen frac-

<sup>3</sup> Glucose- $U-C^{14}$  signifies glucose uniformly labelled with  $C^{14}$ .

tions of the brain indicate that the free glycogen is most actively synthesized and is probably the common source of glycogen in the other fractions. Kolotilova (47) reported that the investigation of reaction rates of individual steps in the processes of anaerobic cleavage of sugar to lactic acid in muscle and nonnucleated erythrocytes revealed that the over-all rate of the glycolytic process is proportional to the rate of the slowest reaction in the process. Among the factors which determine the rate of glycolysis is the concentration of substrates and cosubstrates-coenzymes, the concentration of which in tissues is subject to considerable variations. Particularly important in this connection are ATP and ADP, as well as the reduced and the oxidized forms of DPN. The presence of ATP determines the initial reactions of glucose metabolism in tissues. The ratio of DPN to DPNH exerts a regulating effect on the speed and direction of the glycolytic process. Alterations in the rates of individual slow intermediary reactions, which are interconnected via the systems of coenzymes-cosubstrates, are the basic reasons for the alterations in the over-all rate of glycolysis during functional alterations in tissue metabolism. It appears to the author that the investigation of activities of crystalline preparations of individual enzymes of the glycolytic complex is insufficient for the elucidation of chemical mechanisms of changes in the over-all reaction rate of this process in the animal organism. Tracer studies of the individual intermediary reactions of glycolysis would be a better approach.

Shemanova & Blagoveschenskii (48) reported that *Cl. oedemantiens*, type A, contains an enzyme named glycerol kinase which utilizes ATP to phosphorylate glycerol. The optimal pH is 6.9 to 7.1.  $\text{Ni}^{++}$  and  $\text{Mg}^{++}$  equally activate the enzyme,  $\text{Ca}^{++}$  and  $\text{Zn}^{++}$  inhibit it, and  $\text{Mn}^{++}$  is almost inactive. NaF, in a concentration of 0.1 M, does not inactivate the enzyme. In the presence of  $\text{Mg}^{++}$ , fluoride is inhibitory. As in the case of enolase, a possible formation of Mg-fluorophosphate complex is indicated. The presence of glycerol kinase was detectable only in organisms grown and transferred on a glucose medium. Contrary to Oakley *et al.* (49), type B and type A *Cl. oedemantiens* contain glycerol kinase. Glycerophosphate and phosphoglyceric acid comprised the greater portion of the phosphorylated compounds. Fructosediphosphate and lactic acid were among the other products found on growing the bacterium on glycerol.

Engelhardt & Kanopkaite (50), employing two systems, carboxylase and pyruvodehydrogenase, showed that the thiazole and sulfhydryl forms of thiaminepyrophosphate are equally active in simple and oxidative decarboxylation systems. The disulfide form of thiaminepyrophosphate, unless first reduced by cysteine, is inactive in both systems. These data do not support the hypothesis of Zima *et al.* (51, 52) to the effect that in the course of its coenzymatic functions thiaminepyrophosphate passes from the thiol to the disulfide form. Lichstein (53) has demonstrated the presence of biotin in purified preparations of oxalacetic carboxylase. Sytinskaya (54) examined the P:O ratio in the liver extracts of biotin-deficient chicks and found it to

be low; 90 min. after the administration of biotin to deficient birds the P:O ratio was raised. The CoA content and activity (acetylation of streptocide) were not affected by biotin deficiency. The site of involvement of biotin in the process of oxidative phosphorylation is under study.

Shapot & Pruss (55) studied the role of oxidative phosphorylation in the function of the isolated, perfused frog heart. Perfusion with 2,4-dinitrophenol ( $1 \times 10^{-3}$  M) gradually weakened heart contraction and finally stopped it. Increasing the "work load" from a pressure of 10 cm. to 30 cm. caused the inhibition of contraction by DNP to appear sooner. It appeared that the greater was the energy expenditure the sharper was the incompleteness of both tissue respiration and ATP resynthesis. That this is indeed the case was shown by the addition of ATP ( $6 \times 10^{-3}$  M) along with DNP to the perfused heart; contraction continued; when the perfusion fluid containing DNP was flushed out with Ringer solution containing ATP, to remove DNP, the contraction of the heart was normal. The inhibition of respiration with cyanide ( $1 \times 10^{-3}$  M) completely inhibited heart contraction and the addition of ATP restored the function. These observations are in line with the suggestion made by Shapot (56), that the true criteria of physiological effectiveness of energy metabolism is not the intensity of tissue respiration or glycolysis, but the capacity of the tissues to resynthesize the energy-rich phosphorus-containing compounds in relation to their need by the tissues. ATP and its resynthesis, via the process of oxidative phosphorylation, play a decisive role in heart function.

Slobodkina (57) reported that sodium bisulfite inhibited glycolysis in the brain tissue by 25 to 32 per cent, in the kidney cortex by 19 to 29 per cent, and not at all in other tissues examined. Turpaev & Mamedova (58), employing  $\text{Cd}^{113}$ , found that the inhibition by Cd of the contractile properties of frog heart occurred when Cd accumulated to 30 to 40  $\mu\text{g}$ . per gm. of tissue. This amount of Cd blocks about 0.35 per cent of free thiol groups of the tissue. In contrast to Cd,  $\text{AgNO}_3$  inhibits the contractile properties of frog heart when about 1 per cent of free thiol groups of the tissue are blocked. Ag, like monoiodoacetate, exerts its effect on heart muscle by inhibiting metabolic reactions which are related to muscular contraction, blocking mainly the glycolytic reactions. Cd, on the other hand, acts on the surface of the muscle tissue, affecting the membrane permeability which depends on the free thiol groups (59). In support of the latter hypothesis,  $\text{Cd}^{113}$  was found to be concentrated principally in the insoluble portion of the heart muscle, part of which is comprised of cell membranes. Fedorov (60) reviewed the modern concepts of the biochemical basis of respiration in microorganisms and came to the conclusion that none of the theories proposed by individual authors explains the entire scope of the chemistry of respiration, although each theory contributes to the development of the modern, much more complex, concept that is not yet complete. Cernoch (61) and Szafranski (62) reviewed the latest developments in the role of the pentose cycle in glycolysis as well as in the energetics of the process.

As for riboflavin, Verbitskaya & Skulskaya (63) found 7 to 12  $\mu\text{g.}$  of this substance per gm. of brain in sturgeon (*Huso huso*) and 5 to 7  $\mu\text{g.}$  per gm. of brain in frogs. These values were the highest found among the 26 species of vertebrates examined. Biochemically, frog brain is characterized by being more primitive in its energy metabolism, namely, high anaerobic glycolysis and low respiration, low activity of the cytochrome-oxidase system, greater relative cyanide-resistant oxidation, and high riboflavin content. These observations suggest that the participation of flavoproteins as the terminal step in the fundamental chain of oxidative reactions in the brain represents a primitive biological type of oxidation, present mainly in species which for one reason or another ceased to evolve.

Epshtein (64) reports that the process of HCl secretion by mucous membrane of the stomach is an active one which proceeds under normal conditions only aerobically and utilizes the basic biochemical mechanisms. Although under certain conditions mucous membrane of the stomach exhibits anaerobic glycolysis, this mechanism of sugar utilization is not basic for the secretion of HCl by the stomach membrane. In a state of active HCl secretion the mucous membrane of the stomach switches over to a more economical apotomic oxidation of sugars (pentose path). Such a switch from dichotomous to apotomous degradation of sugars is true for lactating mammary gland and is fundamental for the activity of such glands as the adrenal. The energy of ATP formed during the oxidative decomposition of sugar is utilized by the stomach membrane for its activity. The energy utilization by the membrane proceeds only in the presence of phosphoproteins and RNA, the latter apparently participating in some fashion in the secretion of HCl as well as in other processes. For the simultaneous liberation of  $\text{H}^+$  and  $\text{Cl}^-$  from blood plasma it is not essential, as has been assumed by Hollander (65), that the membrane of the intracellular canals of surface cells be impermeable to  $\text{Na}^+$  of NaCl.

*Nucleic acids, purines.*—Kotel'nikova (66) reviewed the available data on the interconversion of ribonucleotides, the relationship between free ribonucleotides and RNA, the nature, transformations, and the role of adenosine phosphoric acids, inosine phosphoric acids, uridine phosphoric acids, and cytidine phosphoric acids, as well as the synthesis of free ribonucleotides. Kusin (67) came to the conclusion that the main step in the biological action of ionizing radiation is an effect on polymeric nucleoproteins and nucleic acids, leading to the appearance of abnormal split products of these vital polymers. Alterations in the structure of nucleic acids are reflected in the synthesis of specific protein-enzymes, which, in turn, lead to interdependent alterations in individual metabolic reactions. The alterations in the metabolic reactions are accentuated by changes in the adsorption of enzymes on the surfaces of microstructures and by changes in permeability, which are a result of depolymerization of complex proteins of high molecular weight present in living tissues.

Spirin *et al.* (68) grew *E. coli* on synthetic media and the composition of



the cells was examined at 10, 20, and 30 hr. of growth for DNA, RNA, and their components by a procedure described previously (69). The DNA was examined by the procedure of Marshak & Vogel (70), except that extraction with hot TCA was replaced by extraction with perchloric acid (71). The composition of RNA and DNA did not alter during growth, i.e. there was no age specificity with respect to either nucleic acid. The content of RNA decreased with age from 9.6 per cent at 10 hr. to 5.3 per cent at 30 hr. The content of DNA (2.4 per cent of dry weight of bacteria), on the other hand, was the same at all ages. The data are in accord with those of Elson *et al.* (72), Reddi (73), and Dutta *et al.* (74), suggesting an absence of age specificity in nucleic acid composition in a wide variety of organisms. Davidson *et al.* (75) studied the incorporation mechanisms in nucleic acid biosynthesis. Whereas, *in vivo*, bone marrow and ascites cells of tumors intensively incorporated formate- $C^{14}$  into the purines of nucleic acids, *in vitro* the same tissues retained the ability to incorporate formate- $C^{14}$  into only thymine of DNA, but lost the capacity to incorporate it into purines. On addition of an extract of rat or mouse liver, bone marrow and the ascites tumor cells regained the capacity to incorporate radioformate into purines *in vitro*. Tissues of thyroid gland of rabbit, mouse carcinoma 2146, and MCLM-ascites carcinoma also incorporated radioformate more intensively into thymine DNA than into purines *in vitro*, and, on the addition of a liver extract to the tissues, the incorporation of radioformate *in vitro* into purines increased. The surviving cultures of Earl, line "L," grown in salt solution in the presence of 30 per cent horse serum and 10 per cent embryonic extract (containing preformed purines), behaved in a manner similar to bone marrow and ascites cells *in vitro*, and the addition of a liver extract to the cultures increased the synthesis of purines from formate. If purines were omitted from the medium in which the "L" cells were grown, then the extent of incorporation of radioformate into purines was about the same as into thymine. On addition of adenosine or guanosine to the medium the neosynthesis of purines from formate was depressed. These observations suggest that some rapidly growing tissues do not normally synthesize by themselves all the purines they require for nucleic acid synthesis but in the intact animal depend on the assistance of other tissues, particularly the liver. When removed from the animal body and incubated *in vitro*, such tissues continue to synthesize nucleic acids, utilizing (in addition to such limited amounts of purines as they synthesize *de novo*) preformed purines already present within the cells.

In the course of an investigation of the metabolism of Ehrlich ascites cells, Straub *et al.* (76) have observed that these cells, in contrast to normal tissue cells, show a low activity of adenosine deaminase; the ascitic fluid, however, shows a relatively high activity. On injection of Ehrlich ascites cells into mice, the adenosine deaminase activity of blood plasma rose three- to fourfold. In human tumor-bearing patients the adenosine deaminase activity of blood plasma was also elevated. The enzyme, present in the ascitic fluid and in the blood plasma of Ehrlich ascites-bearing mice, originates from



tumors, although the tumor cells themselves have a low adenosine deaminase activity. That the tumor cells actually produce the enzyme was demonstrated. A suspension of the washed ascites cells in Krebs phosphate saline buffer containing casein hydrolysate was placed in a cellophane sac intra-abdominally in a normal mouse and kept there for 24 hr. Part of the suspension was kept at 0° as a control. The latter showed no increase in adenosine deaminase activity, while the fluid and cells which were kept intra-abdominally in healthy mice showed a large increase in the adenosine deaminase activity of the fluid but not in that of the cells. The measurement of adenosine deaminase activity in human blood plasma was proposed as a clinical test for diagnosis of possible malignancy (77).

Vilenkina (78) isolated from the urine of normal humans 4,5-amino-5,4-imidazole carboxamide which proved to be identical with the product formed by cultures of *E. coli* grown in media supplemented with sulfathiazole (79). Kritskii (80) observed that the addition of DNA to bone marrow preparations of pigeons and rabbits sharply increased the incorporation of P<sup>32</sup> into a chromatographic component of undefined nature. Incubation of bone marrow homogenates with DNA in a phosphate buffer sharply induced the production of nucleotides from DNA. Apparently under these conditions, DNA activates a reaction or reactions which not only bind inorganic phosphate but at the same time induce cleavage of DNA to polynucleotides. In this connection it is of interest that polynucleotides appear to play a prominent role in oxidative phosphorylation (81).

*Amino acids, enzymology.*—Akabori *et al.* (82) prepared an asymmetric catalyst from silk fibroin and palladium black, and by employing it were able to reduce diethyl- $\alpha$ -acetoximinoglutarate to L-(+)-glutamic acid, ethyl- $\alpha$ -acetoximinophenylpropionate, and  $\alpha$ -acetaminosuccinic acid; 4-benzylidene-2-methyloxazol-5-one was reduced to L-phenylalanine in good yields. These experiments suggest that palladium black, adsorbed on a supporter with an asymmetric configuration, is capable of catalyzing an asymmetric reduction. Proskuryakov & Buachidze (83) find that malate and glucose-1-phosphate act as the hydrogen donors in either the aerobic or anaerobic reduction of DPN to DPNH, the cofactor of cystine reductase. These studies confirm those of Nickerson & Romano (84), who found that the system which reduces cystine to cysteine but does not attack oxidized glutathione involves DPNH and systems which reduce DPN to DPNH. Rall & Lehninger (85) and Anderson *et al.* (86) have previously shown that in the system which reduces glutathione the hydrogen donors are glucose-1-phosphate, isocitrate, and malate.

Trufanov & Popova (87) report that the synthesis of coenzyme-A from pantothenate occurs in benign tumor tissue but not in malignant tumors (medulloblastoma, sarcoma). When pantothenate was substituted by pantotheine, both benign and malignant tumors synthesized CoA. Apparently in the malignant tumors, systems which synthesize the peptide bond of pantotheine are inoperative.

Kaganova & Orekhovich (88), using chymotrypsin, have shown that the ethyl ether of tyrosine is a convenient substrate for studies of transpeptidation. The same authors (89) now report that synthesis of tyrosine peptides occurs along with hydrolysis of tyrosine ether in aqueous extracts of kidney and liver of guinea pig, rat, and swine kidney at pH 5. Free tyrosine, when incubated under similar conditions, was ineffective in this respect. The compound, by isolation and analysis according to Sanger (90) was either a dipeptide of tyrosine or a dipeptide of tyrosine ether. Incubation of tyrosine ether with glycine, leucine, glycylglycine, leucylglycine, glycylytyrosine, and leucylglycylglycine gave rise to new peptides only with glycylglycine, glycylytyrosine, and leucylglycine. Analysis of the new peptides indicated that in all cases the N-terminal amino acid was tyrosine. The  $R_f$  values of the new peptides was the same as for peptides which were synthesized from tyrosine ether and the above mentioned peptides in the presence of chymotrypsin: tyrosylglycylglycine, tyrosylleucylglycine, and tyrosylglycylytyrosine. Cathepsin-C, prepared from ox spleen (91), possessed high proteolytic and transpeptidase activities. Purified cathepsin, however, possessed only the proteolytic activity, was free of esterase and transpeptidase activity, and did not cleave tyrosine ether. These findings are in accord with earlier observations (92). The data, therefore, suggest that the enzyme responsible for transpeptidation is not cathepsin-C but an esterase which cleaves tyrosine ether. By means of electrophoresis on starch at pH 8.6 in veronal buffer, esterase was obtained with was not specific for tyrosine ether. The specific esterase was probably adsorbed on the starch during electrophoresis, since transpeptidase activity was lost from extracts which had passed through a starch column. Transpeptidase activity was retained after saturation of the starch with a 1 per cent solution of NaCl in phosphate buffer (pH 6.8) and a 1 per cent solution of NaCl in borate buffer (pH 7.45). The authors conclude that transpeptidation occurs along with hydrolysis of tyrosine ether. Cathepsin-C did not hydrolyze tyrosine ether, and purified preparations of cathepsin-C and esterase did not have transpeptidase activity.

Kaplanskii & Kaplanskaya (93) observed that protein deficiency in the diet of rats leads to lowered oxidation of tyrosine and phenylalanine by the liver. Later, Kaplanskii & Mashbiz (94) suggested that the reason for the lowered oxidation of these two amino acids was endogenous avitaminosis-C, which in rats always accompanies protein deficiency. Sealock & Silberstein (95) previously demonstrated the involvement of ascorbic acid in the oxidation of tyrosine in the liver. Kasantseva & Kaplanskii (96) have now showed that the oxidation of tyrosine by slices or homogenates of livers from rats kept on low-protein diets was reduced three to fourfold, and that the addition of ascorbic acid or glutathione to liver preparations did not improve the oxidation of tyrosine. The addition of ketoglutarate, alone or with ascorbic acid, markedly improved the oxidation of tyrosine. Further addition of glutathione was ineffective. Addition of citric acid to liver homogenates from normal rats improved the oxidation of tyrosine, whereas the addition of citric

acid to liver homogenates from deficient rats did not. It is concluded that a protein deficiency in rats impairs the oxidation of citrate to ketoglutarate, and the lowered rate of ketoglutarate formation in the deficient rats impairs the first step in the oxidation of tyrosine, its deamination via transamination.

Kaplanskii *et al.* (97) further report that the extent of deamination of L-alanine is about one-half that of DL-alanine in slices of liver from normal rats. The addition of ammonia inhibits the deamination of L-alanine but has no effect on the deamination of D-alanine. A portion of the ammonia liberated after deamination of L- and DL-alanine is utilized in the presence of ketoglutarate for the synthesis of glutamine. In protein-deficient rats, the extent of deamination of L-alanine is reduced by 50 per cent. Beresovskaya & Smirnova (98) report that on a diet deficient in protein (3 per cent) the synthesis of alanine, glutamic acid, and aspartic acid from the corresponding keto acids by liver slices was decreased, the rate of amination of pyruvate and oxalacetate being depressed considerably more than the rate of amination of ketoglutarate. The rate of transamination between alanine and ketoglutarate was reduced by 30 per cent, and that between aspartic acid and ketoglutarate by about 25 per cent. In confirmation of the results of Awapara (99), alanine-ketoglutaric transamination was markedly depressed after about four to five days on the 3 per cent protein diet. The amination of ketoacids has previously been found to be unaffected by low protein diets (100), but the opposite results reported in this study are due to the considerably longer time of maintenance on the low protein diet with an attendant 25 to 30 per cent loss in weight. Beresovskaya (101) has shown that the synthesis by cell fractions of amino acid nitrogen from pyruvate and ammonia occurs mainly in mitochondria. The nuclei contain a factor which stimulates the production of amino acid N. These experiments, however, do not show whether this synthesis of "amino acids" is a result of direct amination of pyruvate or whether, as proposed by Braunshtein (102), it is a result of transamination. In these experiments ATP protected mitochondria from destruction, but it did not stimulate the synthetic process, which occurs anaerobically or aerobically.

Gershenovich & Krichevskaya (103) find that the glutamine synthetase activity of brain is more resistant to hyperoxia than that of liver, although the activity of both enzymes is reduced by increased oxygen tension. Ferdman & Silakova (104) find that the stimulation of skeletal and cardiac muscle increases the content of ammonia and glutamic acid with a concomitant decrease in glutamine amide N; a similar observation was noted on stimulation of rabbit muscle *in situ* with electric current. Glutaminase activity markedly increases in skeletal muscle on exercise, and returns to normal after a rest period. It is suggested that glutamine is a source of ammonia in muscle. On aerobic incubation of muscle in phosphate buffer at pH 7.2 there is a loss of glutamine amide N, an increase in purine N, and no free ammonia. Increase in purine N does not account for all of the loss of glutamine amide N. The rate of renewal of glutamine amide N was studied

with  $N^{14}$ , and it was found to be greater than that for protein amide N, the nonprotein or the total protein N. Ammonia is presumed to enter the metabolic reactions which involve nitrogenous constituents via glutamine formation. Rasina (105) employed histidine- $C^{14}$  and  $\beta$ -alanine and found that carnosine is synthesized in muscle tissue and not in the liver of young rabbits, in accord with Yudaev (106) and contrary to others (107, 108). Sokolova (109) reports that in guinea pigs the activity of both heart and skeletal muscle ATPase is markedly decreased by avitaminosis C, and is restored to normal by the administration of the vitamin to scorbutic animals.

Luganova *et al.* (110) described experiments in which intact leucocytes demonstrated a high capacity to liberate inorganic phosphate from ATP. After incubation of leucocytes with ATP and phosphate- $P^{32}$ , the isolated ATP (extracellular) was free of activity. This finding suggests that the net cleavage of ATP by leucocytes is not a result of a complex series of reactions taking place on the surface of the cells. The cleavage of ATP is due to either the ecto-ATPase [surface-located ATPase (111)], or the utilization of energy-rich ATP residues without a corresponding resynthesis of ATP. Granulocytes, as well as the lymphocytes and myoblasts, were also capable of cleaving ATP. The cells were unable to liberate phosphate from glucose-1-phosphate, fructose-1,6-diphosphate, 3-phosphoglyceric acid, adenylic acid, cozymase, or inorganic pyrophosphate. The enzyme located on the surface of these cells was more active in normal serum than in various buffers. The enzyme was inhibited by 0.01 *M* K and 0.02 *M* fluoride, activated by 0.01 *M*  $Mg^{++}$ , and unaffected by 0.002 *M* bromacetate. The pH optimum was between 9 and 10. Warming at 45° for 10 min. lowered activity by 60 per cent, and at 50° the activity was lowered by 85 per cent. At 1° the activity was not lost after three days. The capacity of the cells to cleave ATP is high: the amount of ATP cleaved in 4 to 5 hr. was equal to the dry weight of the cells.

Belousova (112) reported that the addition of hydrolysates of RNA to slices of rat liver, spleen, or kidney increased the extent of incorporation of glycine-1- $C^{14}$  and methionine- $S^{35}$  into proteins by 35 to 192 per cent. Perevoschikova & Zbarskii (113) found that slices of sarcoma  $M_1$  and sarcoma 45 incorporated the isotopic amino acids into proteins more intensively than did the normal tissues, but the addition of the hydrolysates of RNA to slices of malignant tissues had only a negligible effect. Ehrlich ascites cells, however, incorporated markedly greater amounts of the radio-amino acids into proteins on addition of the RNA hydrolysate. Sonic vibrations of the cells decreased the extent of incorporation of radioamino acids by fifteen- to twentyfold, and the addition of the RNA hydrolysate to the cells so treated sharply increased the incorporation into proteins. In accordance with the hypothesis of Gale & Folkes (114), it appears that as long as DNA is preserved in the systems employed the products of RNA hydrolysis may serve for specific nucleoprotein synthesis. Whether the biosynthesis of protein takes place simultaneously with the synthesis of

nucleic acids, or whether these two processes are to a certain extent antagonistic to each other is of interest, particularly because the nuclei of malignant cells possess a high capacity for the synthesis of nucleic acids and a relatively low capacity for the synthesis of protein (115, 116).

According to Braunshtein (117), indirect metabolic processes, mediated through transamination reactions, play a predominant role in the physiological dissimilation of nitrogen in mammalian protein metabolism. Kluge (118) has now shown that in rat liver homogenates, fortified with ATP and fumarate, and stabilized with versene, a variety of L- and D-amino acids and ammonia can serve as efficient N-donors in the conversion of citrulline to urea. Urea formation from citrulline and various amino acids is not impaired in liver homogenates of pyridoxine-deficient rats, due to the high residual activity of aminophosphatases. Inhibition of transamination reactions with  $\text{NH}_2\text{OH}$  and isoniazid resulted in the blockage of urea formation from citrulline and all N-donors ( $\text{NH}_3$  and amino acids) with the exception of L-aspartate.  $\alpha$ -Methylaspartate inhibited urea synthesis from citrulline and all N-donors including aspartate. As shown by Braunshtein *et al.* (119),  $\alpha$ -methyl-aspartate acts as an antimetabolite in the condensation of aspartic acid and citrulline. When urea synthesis is inhibited by carbonyl poisons or by  $\alpha$ -methyl-aspartate, or by omission of citrulline or fumarate, liver homogenates liberate ammonia from D-amino acids, but not from glycine nor from any of the L-amino acids tested. Inhibition of D-amino acid oxidase and glycine oxidase by Na benzoate failed to impair urea synthesis from glycine in the second stage of the ornithine cycle, while it depressed the formation of ammonia and urea from D-amino acids.

Several amino acids, such as histidine and serine, may undergo an oxidative deamination prior to utilization of their N for urea synthesis. Upon inhibition of histidine deaminase with ethylenediaminetetracetate, transamination occurs; this is considered as an alternative pathway for the formation of urea from histidine and aspartic acid. The conclusion is that all N-donors react with citrulline in the second stage of urea formation only via the conversion to aspartic acid, as suggested by Ratner & Pappas (120). Glycine and most L-amino acids are not deaminated in liver tissue at physiological concentrations. Aspartic acid and urea are formed from these amino acids in the liver almost exclusively via transamination, and from ammonia only by transamination via glutamate (121, 122). D-Amino acids are oxidatively deaminated to give ammonia, and some amino acids particularly may undergo anaerobic deaminative cleavage. The inhibition of ketoglutarate formation by fluoroacetate suggests that the two-step transamination via glutamate plays a predominant role in the conversion of monocarboxylic acids to aspartic acid in liver tissue. It is further inferred that, in mammalian protein catabolism, almost 50 per cent of N can be dissimilated and converted to urea via transfer reactions without the intermediary formation of  $\text{NH}_3$ . The major part of the ammonia, which supplies the second moiety of urea in the first stage of the ornithine cycle, must

originate from direct or indirect deamination processes in extrahepatic tissues. It is probable that this ammonia is conveyed to the liver by the bloodstream as the amide group of glutamine.

On the basis of the data of Kluge (118) on the inhibition of the synthesis of urea from citrulline and all N-donors by  $\alpha$ -methyl-aspartic acid in liver homogenates, it appeared possible to Braunshtein *et al.* (123) that the effect of the  $\alpha$ -substituted aspartic acid is due to the inhibition of synthesis of argininosuccinic acid and citrulline in the second phase of the ornithine cycle. In accord with the theory of Ratner (120), Kluge (118) believes this reaction to be the necessary intermediate step in the utilization of N from all amino acids and ammonia for the conversion of citrulline to arginine (and urea). Braunshtein *et al.* (123) have now shown that  $\alpha$ -methyl-aspartic acid selectively inhibits the synthesis of urea in liver slices from ammonia, and in homogenates from citrulline and aspartic acid or other N-donors, but it has no inhibitory effect on the formation of citrulline from ornithine,  $\text{CO}_2$ , and  $\text{NH}_3$ , the cleavage of argininosuccinic acid or the activity of arginase.  $\alpha$ -Methyl-aspartic does not affect transamination reactions, cell respiration, or oxidative phosphorylation. This acid, as a structural analogue of aspartic acid, inhibits the condensation of aspartic acid with citrulline. The inhibition of the second phase of the ornithine cycle by  $\alpha$ -methyl-aspartic acid is determined mainly by the concentration of the acid and is independent of the concentration of aspartic acid, citrulline, or respiratory substrate. The inhibition is increased by preincubation of liver homogenates with  $\alpha$ -methyl-aspartate.

Shemiyaikin & Schukina (124) presented a theory of oxidative-hydrolytic transformations of organic molecules, and a comprehensive collection of data is offered in support of the main points of the theory of oxidative-hydrolytic cleavage of C—C bonds in biological systems.

*Methodology.*—Procedures were described for the determination of cholesterol in blood (125), cell substances (cellulose) (126), thiamine in blood (127), adenine in tissues by a polarographic method (128), thyroxine and triiodothyronine (129). A new method for the isolation and purification of cytochrome-*c* was described (130).

*Miscellaneous Studies.*—Yudaev & Druzhinina (131) have previously shown that adrenals of newborn animals contain enzymes which catalyze the synthesis of corticosteroids. This observation suggested the possibility that the newborn animal is capable of converting  $\text{C}_{19}$  to  $\text{C}_{21}$  corticosteroids. The same authors (132) incubated dehydroisoandrosterone with slices of adrenal cortex of three- to ten-day-old pigs. The steroids were extracted from the incubation mixture with ethyl acetate, purified (133), and analyzed chromatographically (134). Three  $\text{C}_{21}$  corticosteroids and one  $\text{C}_{19}$  corticosteroid were obtained. Two of the  $\text{C}_{21}$  steroids were 17-oxycorticosterone and corticosterone; the third  $\text{C}_{21}$  steroid was less polar than the 17-oxycorticosterone, and it contained the characteristic  $\Delta^4$ -3-keto group in ring A and a keto side chain at  $\text{C}_{17}$ . It did not contain an hydroxy group at  $\text{C}_{17}$ . The



C<sub>11</sub> steroid, which accumulated in largest amounts, was identified as  $\Delta^4$ -androstan-11- $\beta$ -ol-3,17-dione. The capacity to transform C<sub>19</sub> to C<sub>21</sub> steroids is not peculiar to newborn tissue. Adrenal cortex of adult swine is also capable of this transformation. A scheme proposed for the biosynthesis of steroid hormones includes a pathway via C<sub>11</sub> steroids (Fig. 1).

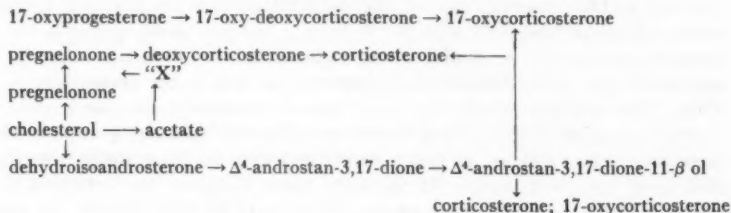


FIG. 1. Biosynthesis of steroid Hormones.

Khokhlov *et al.* (135) described a detailed method for the preparation of chemically pure phenoxymethyl-penicilline and its Na,K, procaine, and N,N'-dibenzylethylenediamine salts. Silaev & Stepanov (136) studied the significance in gramicidine of the free amino group which imparts biological activity. The authors prepared N-phthaloyl-gramicidine, N-guanyl-gramicidine, N-succinyl-gramicidine, N-carbamyl-gramicidine, and N-nitro-guanyl-gramicidine. Only the N-guanyl-gramicidine retained full activity against *S. aureus*, suggesting that the change of the ornithine moiety of gramicidine to arginine does not alter biological activity and indicating that possibly the N-guanyl substituted gramicidine underwent biological transformation to the original structure (arginine moiety to ornithine moiety). It would be of interest to determine if any of the other N-substituted gramicidines or gramicidine itself is involved in the bioreactions involving ornithine. Saev & Markov (137) observed that *S. aureus* in West-Wilson medium does not develop if only nicotinic acid is added to the culture (41 strains). If thiamine or the imidazole and the pyrimidine moieties of thiamine are also added growth occurs. In 155 strains tested, the thiamine moiety could be replaced by an equivalent amount of penicillin or benzylpenicilloic acid. This was true only for penicillin-resistant strains.

It has been previously reported that various substances which can be considered structurally and genetically related to carotene possess physiological activity which is directed against the activity of humoral mediators, such as acetylcholine and histamine (138 to 141). Drosdova *et al.* (142) now report that antihistamine and antiacetylcholine effects were produced with an aqueous colloidal solution of oxidized carotene. After the removal of the oxidized carotene from the site of action, the tissues (guinea pig intestinal segments) retained their sensitivity to histamine and acetylcholine.

Soldatenkov & Masurova (143) found that kidney beans and clover contain malonic acid in an amount equal to 45 per cent of the total content of



di- and tricarboxylic acids; this is equivalent to 2 to 3 per cent of the dry weight of the plant. In view of the known inhibitory effects of malonic acid on the transformation of succinic acid to fumaric acid and on the activity of succinodehydrogenase, these concentrations of malonic acid would exclude or limit the operation of the Krebs cycle in these plants. Kursanov & Kriukova (144) observed that during the infiltration of the leaves of kidney beans (*Phaseolus vulgaris*) with 0.1 M keto or hydroxy acids (pyruvic, ketoglutaric, oxalacetic, or malic) photosynthesis in the absence of free CO<sub>2</sub> occurred very slowly (about 6 to 7 per cent of that in the presence of free CO<sub>2</sub>). This indicates a relatively poor rate of decarboxylation, and hence a limited capacity of bean plant leaves to utilize the carboxyl groups of the keto and hydroxy acids. In this respect the leaves of beans differ from the tissues of succulent plants. At the same time, however, the keto and hydroxy acids of the Krebs cycle are able to activate by more than 50 per cent the photosynthesis which occurs in the presence of free CO<sub>2</sub>. This effect is more or less specific for these acids, since lactic, butyric, ascorbic, oxalic, acetic, and formic acids did not stimulate photosynthesis under similar conditions.

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